When the amplified region of 726 bp was segregated into three regions (I–III), there was no apparent difference between methylation in region I in the kidney and that in region I in the liver. In region II, CpGs in the kidney were slightly methylated compared with those in the liver. In region III, which is included in the CpG island of the gene, CpGs in the kidney were more methylated than those in the liver. Hence, methylation in region III plays an important role in the difference between *CES1A1* gene expression in the kidney and that in the liver, and methylation in region II may also have a minor role in that difference. It was previously suggested that methylated and inactive promoters are occupied by nucleosomes in the silenced state of the *MLH1* gene (Lin et al. 2007). Nucleosomal occupancy is involved in methyl-binding proteins (Li et al. 2007). In such a mechanism, *CES1A1* gene expression may also be repressed possibly by methylation in the promoter region, particularly at position −24 immediately upstream of the TSS. There was partial discordance of the methylation pattern between cell lines and tissues. To explain this phenomenon, we focused on three points. First, the tissues consist of several kinds of cells, whereas the cell lines consist of almost one kind of cell. The hypermethylated clones were observed in the genomes obtained from all liver tissues at almost the same rate. There is a possibility that the hypermethylated clones were obtained from hepatic non-parenchymal cells. Second, there is a possibility that a cell line is partially different from a normal tissue in DNA methylation pattern. Third, although DNA methylation pattern can change with age (Bjornsson et al. 2008), the ages of tissue specimens and cell line samples used in this study were not the same.

Since CESs catalyse the hydrolysis of PNPA, we performed enzyme assay using PNPA. As the result of treatment with 5-aza-dC to HEK293 cells, the activity of hydrolysis of PNPA was increased approximately 25% compared with that of control. But the increases of the activity were lower than expected. We thought that more time after transcription to observe the change caused by single or combined treatment with 5-aza-dC and TSA may be necessary, but after transcription how fast functional CESs are generated is unknown. It is known that the half-life of a rat liver CES isozyme is 42 h (Heymann et al. 1979). Although the half-life of CES1A1 protein is unknown, the finding of half-life for CES1A1 would be explain the difference between the activity of hydrolysis caused by treatment with 5-aza-dC to HEK293 cells and the activity of hydrolysis of HuH-7 cells.

The present study provides information on the metabolism and disposition of prodrugs associated with CES1A1. For example, CES1A1 converts oseltamivir, an inhibitor of viral neuraminidase, into the active metabolite Ro 64-0802, oseltamivir carboxylate, in the human liver (Shi et al. 2006). Oseltamivir can cross the blood–brain barrier (BBB), and its brain penetration at the BBB is limited by P-glycoprotein (P-gp) (Morimoto et al. 2008; Ose et al. 2008). The degree of penetration of Ro 64-0802 at the BBB is lower than that of oseltamivir. According to Ose et al. (2009), Ro 64-0802 in the brain is eliminated across the BBB by its active efflux by multidrug resistance-associated protein 4 (Mrp4, *Abcc4*) and organic anion transporter 3 (Oat3, *Slc22a8*) in mice, although Oat3 may not affect its brain distribution in a steady state. These findings indicate that if *CES1A1* gene expression in the human liver or brain capillary decreases, the distribution of oseltamivir to the brain may increase. It is known that oseltamivir and Ro 64-0802 affect neuronal excitability in rat hippocampal slices (Izumi et al. 2007). Recently, the relationship between abnormal behaviour of children and oseltamivir medication has been studied in detail, but the relationship remains unclear. Yang et al. (2009) revealed that expression level of the human *CES1A* gene in the liver of children is lower than that in the liver of adults and that liver microsomal samples pooled from children showed approximately 15% of the activity of the samples pooled from adults in hydrolysing oseltamivir. Hence, there is a possibility that the difference in *CES1A1* gene expression between children and adults is involved in the abnormal behaviour of children. Considering that DNA methylation pattern can change with age (Bjornsson et al. 2008), we speculated that the difference in *CES1A1* gene expression between children and adults is probably due to the difference in DNA methylation levels in the region around the TSS. This point of view may help to understand the relationship between abnormal behaviour and oseltamivir medication.

The present study revealed that DNA methylation is involved in *CES1A1* gene expression. The difference between the gene expression in the human kidney and that in the human liver may arise from the difference in DNA methylation levels in the region around the TSS.

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Declaration of interest

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Review

(Special Topic)

Carboxylesterases: structure, function and polymorphism in mammals

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This review covers current developments in molecular-based studies of the structure and function of carboxylesterases. To allay the confusion of the classic classification of carboxylesterase isozymes, we propose a novel nomenclature and classification of mammalian carboxylesterases on the basis of molecular properties. Mechanisms of the regulation of the gene expression of carboxylesterases by xenobiotics, and the involvement of carboxylesterase in drug metabolism are also described. The novel biomarker for organophosphate pesticide exposure developed here is much more useful and reliable than cholinesterase inhibition. © Pesticide Science Society of Japan

Keywords: carboxylesterase, genetic polymorphism, molecular structure, classification, novel biomarker.

1. Introduction

The present review highlights the importance of structure in delineating overall function, substrate specificity, regulation and localization of carboxylesterases (CEs). Structural considerations emerge from the genes encoding the family of enzymes. Sequence homology typically yields insights into the evolutionary relationship between the members and the conserved and divergent areas of sequence. Diversity in the structure and ultimately function and cellular localization of the gene product is achieved through gene doubling and divergence, alternative mRNA processing, and post-translational modification.

The acetylcholinesterases (AChE) and CEs belong to a protein superfamily termed the α,β-hydrolase-fold family[1] in which members may have highly specialized functions, as is the case for AChE and juvenile hormone esterase. These members show a high degree of selectivity for a neurotransmitter or a hormone, respectively. Namely, other members of the family, such as butyrylcholinesterase (BuChE) or the wide variety of CEs found in tissues and plants, show greater activity in substrates with which they catalyze. Hence, they serve a protective and clearing function for foreign substrates encountered through the diet or other routes of exposure. This family of enzymes also shows great differences in the cells in which they are expressed; some are found in multiple cell types, whereas others show highly selective expression.

Finally, within the cell itself, we observed distinctive localizations; some of the enzymes are destined for export into the plasma, whereas others are associated with the cell membrane with its catalytic function directed extracellularly. Others may be retained within subcellular organelles, such as the endoplasmic reticulum (ER), whereas still others are found in the cytoplasm.

Unlike the serine superfamily, it is clear that paraoxonases are not serine or cysteine esterases, although we know less about the structure of paraoxonases. Rather, they belong to a discrete family of esterases, most likely those in which a divalent metal is required for catalysis. Considerable progress has been made recently in their purification and structural elucidation. Importantly, inroads also have been made in detecting natural substrates for paraoxonases, and lactones emerge as a prime candidate across several species.

The expression profiles of gene expression encoding esterases are highly regulated during development by nutrition

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status, hormonal factors, and xenobiotics. Although the consequences of regulating esterases by drugs and chemicals have been intensively studied, relatively little is known about the mechanisms by which esterases are regulated by physiological factors. This regulation has several potential consequences for the pharmacological and toxicological actions of drugs and chemicals in humans and animals in different developmental stages and nutritional states.

2. Mammalian CEs

Carboxylesterases (EC 3.1.1.1) are members of an α,β-hydrolase-fold family and are found in various mammalian species.[2-14] These enzymes efficiently catalyze the hydrolysis of a variety of ester- and amide-containing chemicals as well as drugs and chemicals to the respective free acids. They are also involved in the detoxification or metabolic activation of various drugs, pesticides, environmental toxicants and carcinogens. Carboxylesterases catalyze the hydrolysis of endogenous compounds, such as short- and long-chain acylglycerols, long-chain acyl-carnitine, and long-chain acyl-CoA esters.[2,15-22] We have reviewed the characteristics of CEs in relation to the metabolism of xenobiotics.[23-26] Multiple isozymes of hepatic microsomal CE exist in various animal species[27-29] and some of these isozymes are involved in the metabolic activation of certain carcinogens, as well as being associated with hepatocarcinogenesis.[17] It has been suggested that CEs can be classified into five major groups, CES1 to CES5, according to the homology of the amino acid sequence,[23,25,26] and the majority of CEs that have been identified to belong to the CES1 or CES2 family.

Striking species differences have also been shown;[28-30] for example, Inoue *et al.*[31] showed that esterase activity in the dog intestine is very weak and produced no appreciable active band in disk electrophoresis coupled with the staining of esterase activity. On the other hand, esterase activities were observed in the intestines of other species (human,[25,30,31] rat,[30,31] mouse,[31] guinea pig,[31] rabbit,[31] dog[30,31] and monkey[30]) and were found to produce a few active bands in an electrophoretic assay.

It is thought that CEs are one of the major determinants of the pharmacokinetics and pharmacodynamics of ester drugs or ester prodrugs. Since the pharmacological data on ester prodrugs obtained from preclinical experiments are generally used as references for human studies, it is important to clarify the biochemical properties of each CE isozyme, including substrate specificity, tissue distribution, and transcriptional regulation.

This review addresses the significant differences in the molecular structure and function of recently identified CEs, and proposes a novel nomenclature for mammalian CE isozymes based on the nucleotide sequences of the genes encoding the individual isozymes. In addition, the different structure-activity relationships of substrates with each CE family and the genetic polymorphism of CE genes are also described.

3. Novel Classification and Nomenclature of Mammalian CEs

According to the classification of esterase by Aldridge,[33] the serine superfamily of esterase, *i.e.*, AChE, BuChE and CE, falls into the B-esterase group. CE isozymes were initially classified by their substrate specificities and isoelectric point; however, this classification is ambiguous in overlapping substrate specificities. A single esterolytic reaction is frequently mediated by several kinds of enzyme. Recent studies on esterases, as on other enzymes concerned with xenobiotic metabolism, have afforded evidence of multiple forms.

It seems almost impossible to classify these CE isozymes based on their substrate specificity along the lines of the International Union of Biochemistry (I.U.B.) classification, because the individual hydrolases exhibit properties of CE, lipase or both. Mentlein *et al.*[16] proposed classifying these hydrolases as "unidentified CEs" (EC 3.1.99.1 to 3.1.99.x). Based on the high homology and similarity of the amino acid sequence alignment of the encoding genes, we tried to classify CE isozymes into five families: CES 1, CES 2, CES 3, CES4 and CES 5[24,25] (Fig. 1).

The CES 1 family includes the major form of CE isozymes (more than 60% homology with human CES). Thus, they could be divided into eight subfamilies: CES1A, CES1B, CES1C, CES1D, CES1E, CES1F, CES1G, CES1H. Most of the CES1 families, except CES1G, are mainly expressed in the liver. The CES 1A subfamily includes the major forms of human CEs,[18,19,34,36-38] and the major isoforms of rat,[9] dog,[19] rabbit[37] and mouse[35] CE. The CES1B subfamily includes the major isoforms of rat,[9] mouse[20] and hamster[47] CE, and CES1C includes the major isoforms of dog,[18,19] cat[50] and human[38] CE. The CES 1H subfamily includes RL1 (CES1H4), mouse ES 4 (CES1H1) and hydrolase B (CES1H3) and C (CES1H2), which catalyze long-chain acyl-CoA hydrolysis.[6,9,18,27,39] Members of the CES 1G family are not retained in ER, which are secreted into the blood from the liver,[8,35] and these families are all secretary-type CEs. It is interesting that the CES1G family is found in only rats and mice, but not humans, and they are all secretary types of CESs. Although a high level of CES1 activity is detected in the blood of rats and mice, no activity is detected in human blood.

In contrast, the CES2 family is mainly expressed in the small intestine. It includes human intestinal CE (CES2A1),[34,40-44] rat CES2 (CES2A10),[22] rat intestinal CE RL4 (rCES2) (CES2A6),[45] rabbit form 2[46] and hamster AT51 (CES2A11).[47] CES3 includes ES-male (CES3A2) and human CES3 (CES3A1).[48,49] Human CES3 (CES3A1) has about 40% amino acid sequence identity with both CEA1A1 and CES2A1, and is expressed in the liver and gastrointestinal tract at an extremely low level in comparison with CES1A1 and CES2A1.[49]

The CES4 family includes CE-like urinary excreted protein

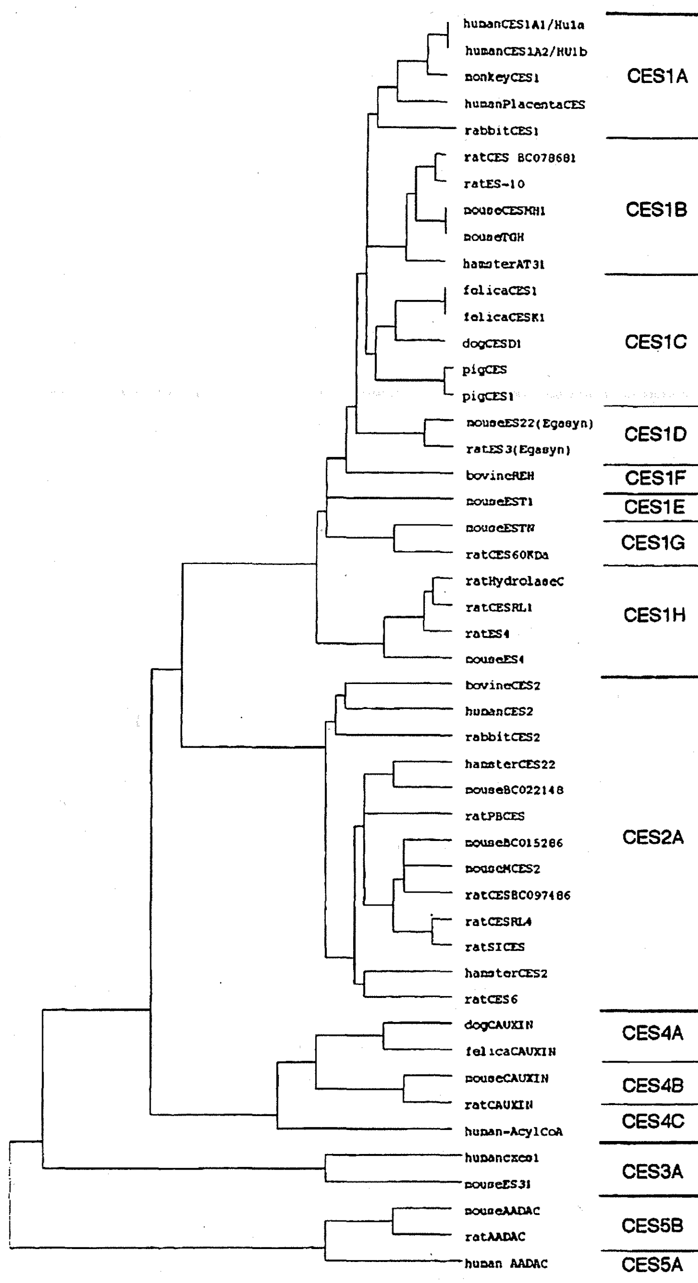

Fig. 1. Phylogenic tree and nomenclature of CE families. Carboxylesterase isozymes are classified into five families, CES1, CES2, CES3, CES4 and CES5. Each family is also divided into subfamilies.

(CAUXIN) (CES4A2), which is excreted as a major urinary protein in cat urine.[50] The CES 5 family includes 46.5-kDa CE isozymes,[51] which have a different structure from the structures of isozymes in other CE families. Esterase (ES) 46.5-kDa from mouse liver[52] and amide hydrolase from monkey liver[10] probably belong to this family. These groupings are similar to the results of phylogenic analysis (Fig. 1).

4. Structure and Catalytic Mechanism of CE Isozymes

It has been shown that several proteins in the ER lumen have a common carboxy-terminal sequence, KDEL-COOH, and

that the structural motif is essential for retention of the protein on the luminal side of the ER through the KDEL receptor bound to the ER membrane.[53–55] Ozols[46] and Korza and Ozols[56] established the primary structures of two microsomal esterases purified from rabbit liver and designated them 60-kDa esterase forms 1 and form 2, respectively. These two forms of CE have a consensus sequence for the ER retention tetra-peptide (HTEL or HIEL in the one-letter code) that is recognized on the luminal side of the KDEL receptor. The HXEL-COOH motif is also essential for retention of the protein on the luminal side of the ER through the KDEL receptor bound to ER membrane.[53–55] Robbi *et al.*[57] reported cDNA cloning of rat liver CES1B4 (ES-10), and that was the first report to show that cDNA of liver CarbE has the consensus sequence of the ER retention tetrapeptide (HVEL-COOH).

Later, Robbi and Beaufay[58] isolated a cDNA clone of another rat liver CES1D2 (ES-3, egasyn), which encoded the consensus sequence of the ER retention tetrapeptide (HTEL-COOH). The other clone, encoded egasyn, is an accessory protein of β-glucuronidase in liver microsomes.[59] Egasyn is identical to CE, and it binds β-glucuronidase *via* its CE active site. In rats and mice, the carboxyl terminal amino acid sequence of clone rat CES-60KDa (CES1G1) and mouse Es-N (CES1G2) is HTEHK-COOH, which can not bind to KDEL receptor, and these isozymes are secreted into blood.[35]

Carboxylesterases have a signal peptide of 17 to 22 amino acid residues near the N-terminal, including hydrophobic amino acid. In the CES1 family, exon 1 encodes a signal peptide.[60,61] In the CES1 family, a bulky aromatic residue (Trp) followed by a small neutral residue (Gly) directly precede the cleavage site.[62] Carboxylesterases have four Cys residues that may be involved in specific disulfide bonds. Among them, Cys98 is the most highly conserved residue in many CE isozymes. Cygler *et al.*[1] reported the important alignment of a collection of related amino acid sequences of esterase, lipase and related proteins based on X-ray structures of *Torpedo californica*AChE and *Geotrichum candidum* lipase.

According to the literature, Ser203, Glu336 and His450 form a catalytic triad, and Gly124-Gly125 may be part of an oxyanion hole (Fig. 2). These residues are also highly conserved among CE isozymes. Site-specific mutation of Ser203 to Thr203, Glu336 to Ala336, or His450 to Ala 450 greatly reduced the CE activity towards substrates; therefore, this mutagenesis confirmed the role of Glu336 and His450 in forming a putative charge relay system with active site Ser203.[24]

Frey *et al.*[63] reported that the formation of low barrier hy-

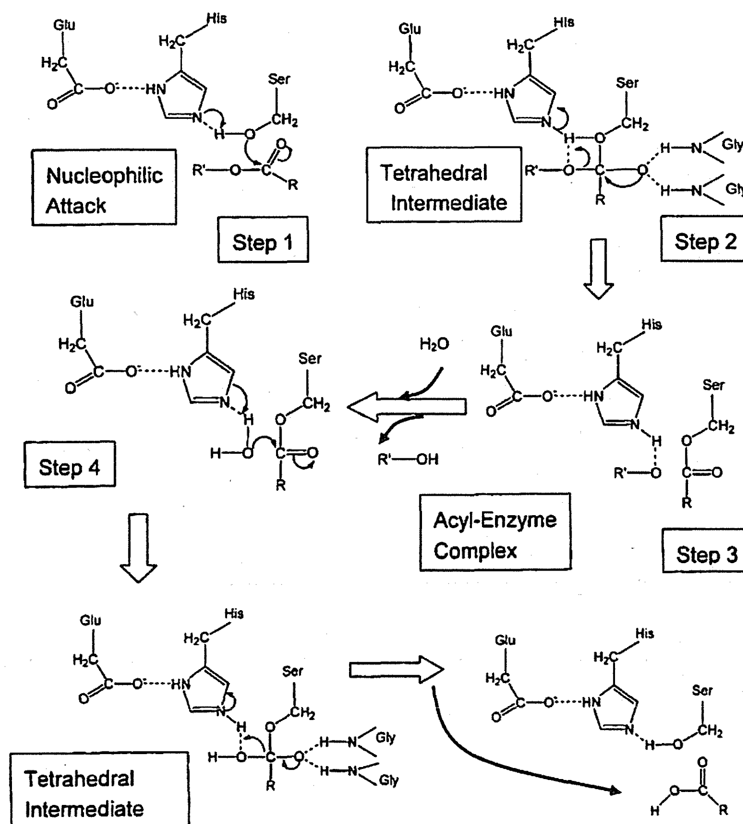

Fig. 2. Proposed mechanism of action of CE. Conformation of the Ser-His-Glu catalytic triad in CE.

drogen bonds between His and Asp (Glu for CE) facilitates nucleophilic attack by the β-OH group of Ser on the acyl carbonyl group of peptide in chymotrypsin. The catalytic triad in the tetrahedral addition intermediate is stabilized by low barrier hydrogen bonds. According to their theory, we speculated that the low barrier hydrogen bond between Glu336 and His 450 facilitates nucleophilic attack by the β-OH group of Ser203 on the carbonyl group of the substrate in CE (Fig. 2).

The mechanism of CE could thus be divided into the following steps: 1) The enzyme substrate complex form, positioning the substrate in the correct orientation for reaction. 2) Hydrolysis of the ester bond starts with an attack by the oxygen atom of the hydroxy group of Ser203 on the carbonyl carbon atom of the ester bond. 3) The hydrogen bonds between the negatively charged oxygen of the tetrahedral intermediate and the N–H group of Gly123 and Gly124 stabilize the negatively charged oxygen (O^-). This configuration, in which negatively charged carboxyl oxygen is hydrogen bonded to two N–H groups, is called an oxyanion hole.

In the general acid-catalyzed step, the ester bond breaks, and the leaving group picks up a proton from the imidazolium ion of His_{450}. The acyl portion of the original ester bond remains bound to the enzyme as an acyl-enzyme intermediate. The alcohol component (R'-OH) diffuses away, completing the acylation stage of the hydrolytic reaction. 4) A water molecule attacks the acyl-enzyme intermediate to give a second *tetrahedral intermediate*. 5) His_{450} then donates the proton to the oxygen atom of Ser203, which then releases the acid component of the substrate. The acid component diffuses away and the enzyme is ready for catalysis.

The tetrahedral transition state is stabilized by the formation of low barrier hydrogen bonds between His450 and Glu336. This low barrier hydrogen bond-facilitated mechanism includes weak hydrogen bonds between the oxyanion (O^-) and peptide N–H bonds contributed by Gly123 and Gly124, which stabilize the tetrahedral adduct on the substrate side of the transition state (Fig. 2). Formation of the acyl-enzyme complex in the next step requires removal of a proton from His_{450}, so that the tetrahedral intermediate is disrupted in the acyl-enzyme intermediate. When the unbound portion of the alcohol group of the first product of the substrate has diffused away, a second step which the deacylation step is essentially the reverse of the acylation step occurs, with a water molecule substituting for the alcohol group of the original substrate.

It is of interest that the sequences required for the hydrolytic capability at the catalytic triad (Glu, His, Ser) of CE, AChE, BuChE, and cholesterol esterase are highly conserved. This is a common structure of α,β-hydrolase-fold families, which are responsible for the hydrolysis of endogenous and exogenous compounds. Furthermore, these elements are strongly conserved among orthologous CEs of the mouse, rat, rabbit, monkey and human.

A three-dimensional model of human CE has been proposed on the basis of crystal structure coordinates of AChE and overlapping active sites with pancreatic lipase and CE.[64] The modeled structure shares the overall folding and topology of the proteins identified in the recently published crystal structures of the rabbit[65] and human CE.[66,67] Carboxylesterase has a three-dimensional α,β-hydrolase-fold structure, which is a structural feature of all lipases.[66] In general, the structure of CE may be viewed as a central catalytic domain surrounded by α,β and regulatory domains.[65,67,68] In essence, the α,β-hydrolase-fold consists of a central β-sheet surrounded by a variable number of β-helices and accommodates a catalytic triad composed of Ser, His and a carboxylic acid. This suggests that the catalytic function of these proteins is conserved across species.

The catalytic triad is located at the bottom from about a 25 Å deep active site, approximately in the center of the molecule, and is composed of a large flexible pocket on one side of Ser203 and a small rigid pocket on the opposite side.[67] The orientation and location of the active site provide an ideal hydrophobic environment for the hydrolysis of a wide variety of hydrophobic substrates.[67] The small rigid active site pocket is adjacent to the oxyanion hole formed by Gly123-124 and is lined by several hydrophobic residues.[67]

Short acyl chains would be easily accommodated within the small rigid pocket. The larger flexible active site pocket is lined by several non-polar residues and could accommodate larger or polycyclic molecules, such as cholesterol. The large pocket is adjacent to a side door secondary pore that would permit small molecules (substrates and reaction products) to enter and exit the active site.[67] Longer acyl chains may be oriented for catalysis in such a way that they extend through the side door. Indeed, the presence of a hydrophobic residue at position 423 in mouse CES1B2 and 425 in human CES1A1 is necessary for efficient hydrolysis of hydrophobic substrates, as a mutation of Met 423 of the related rat lung CE (CES1B4) to Ile increased CE activity towards a more hydrophobic substrate without affecting activity towards short-chain esters.[69]

Most CE isozymes are glycoproteins, and the carbohydrate chain is required for the enzyme activity of CEs.[3,24,26,29,34,68] Human CES2A1 contains a glycosylation site at two different positions (Asn103 and Asn267), while CES1A1 contains only one glycosylation site at Asn79. This glycosylation site is modified by a carbohydrate chain with first N-acetylglucosamine and terminal sialic acid and appears to be involved in the stabilization of the CES1A1 trimer by packing into the adjacent monomer in its crystal structure.[67]

According to the X-ray crystal structure of human CES1, this residue lines the flexible pocket adjacent to the side door.[67] Given the wide range of substrates that CEs are known to hydrolyze, the large flexible pocket confers the ability to hydrolyze many structurally distinct compounds, whereas the rigid pocket is much more selective with regard to the substrates that may be accommodated.

5. Gene Structure and Regulation of CE Isozymes

Both the murine[26] and human[13,61] CES1 genes span about 30 kb and contain 14 small exons. Recently, sequencing of the mouse and human genomes has been completed, enabling detailed sequence comparisons. Previously published sequences of individual exons, splice junctions, size of the introns and restriction sites within the murine and human CE genes are consistent with their respective genes sequenced by the mouse and human genome projects. Therefore, the organization of the CE gene is evolutionarily conserved in mice and humans. Previous studies have mapped the human CE gene to chromosome 16 at 16q13–q22.1.[3,36] This region is syntenic to a region of mouse chromosome 8 at 8C5. The murine CE Es22[60] and Es-N[35] have been previously mapped to chromosome 8. The completion of the mouse genome sequencing project unambiguously demonstrated that the murine CE gene was located on the minus strand of chromosome 8 at 8C5 in a cluster of six CE genes that span 260.6 kb. These six CE genes are presumed to have originated from repeated gene duplications of a common ancestral gene that encoded a CE,[61] and subsequent evolutionary divergence may occur.

Recently, we have identified a mouse liver microsomal acylcarnitine hydrolase, mCES2, as a member of the CES2 family.[45] It has been revealed that this enzyme is significantly induced by di(2-ethylhexyl)phthalate (DEHP) and shows medium- and long-chain acylcarnitine hydrolase activity[45] In addition, we have found that mCES2 is expressed in various tissues with higher levels of expression in the liver, kidney and small intestine. Subsequently, it was shown that three transcription factors, specificity protein (Sp) 1, Sp3 and upstream stimulatory factor 1, could bind to the promoter region of the mCES2 gene, leading to synergistic transactivation of the promoter.[21] Although this mechanism may explain the ubiquitous tissue expression profiles of mCES2, it is unlikely to contribute to the higher levels of mCES2 expression in the liver, kidney and small intestine; therefore, it is thought that another mechanism controls this tissue-specific transcription of the mCES2 gene.[21]

More recently, we have shown that hepatocyte nuclear factor-4 alpha (HNF-4α) can strongly enhance mCES2 gene transcription and that the involvement of HNF-4α accounts for the high expression level of mCES2 in the liver.[71] These findings are notable when the physiological roles of mCES2 are studied, since HNF-4α is involved in various hepatic functions, such as glucose, cholesterol and drug metabolism. In addition, we found that bile acid can repress mCES2 gene transcription by repressing HNF-4α-mediated transactivation.[71]

In 2008, we isolated and characterized two genes encoding the human CES1A1 (AB119997) and CES1A2 (AB119998), and cloned and sequenced the 5' flanking region of each gene in order to elucidate the structure of the promoter[72] (Fig. 3).

It is noteworthy that both the CES1A1 and CES1A2 genes are located on chromosome 16q13–q22 with a tail-to-tail structure. Comparison of the nucleotide sequences of CES1A1 and CES 1A2 genes revealed about 98% homology in 30 Kbp. There are only six nucleotide differences, resulting in four amino acid differences in the open reading frame, and all of the differences exist in exon 1.

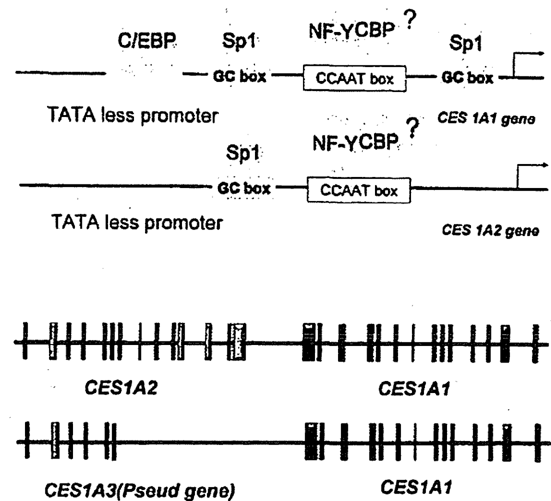

Fig. 3. Structure of the 5' flanking region of CES1A1 and CES1A2 genes. Sp1 and C/EBPa could bind to each responsive element of the CES1A1 promoter but Sp1 and C/EBP could not bind to the 5' flanking region of the CES1A2 promoter. NF-Y, nuclear factor Y; CBF, CCAAT-binding factor

Gene duplication has generally been viewed as a necessary source of material for the origin of evolutionary novelties, and duplicate genes evolve new functions. The majority of gene duplicates are silenced within a few million years, with the small number of survivors subsequently being subjected to strong purifying selection. Although duplicate genes may only rarely evolve new functions, the stochastic silencing of such genes may play a significant role in the passive origin of new species. 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 ER and cytosol.

On the other hand, CES1A2 mRNA was found to be expressed only in the human adult liver, although CES1A1 is expressed in both the human adult and fetal liver.[72] 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 CE genes. Reporter gene assays and electrophoretic mobility shift assays demonstrated that

Table 1. Tissue-specific expression profile of CES1 and CES2 isozymes in mammals and humans.

Species	Isozyme	Liver	Small intestine	Kidney	Lung
Mouse	CES1	+++	−	+++	+++
	CES2	+++	+++	+++	−
Rat	CES1	+++	−	+++	+++
	CES2	−	+++	−	−
Hamster	CES1	+++	−	+++	NT
	CES2	+++	+++	−	NT
Guinea Pig	CES1	+++	+++	++	NT
	CES2	−	+	−	NT
Beagle	CES1	+++	−	NT	+++
	CES2	++	−	NT	+
Monkey	CES1	+++	++	−	NT
	CES2	+	+++	+	NT
Human	CES1	+++	−	+	+++
	CES2	+	+++	+++	−

−, undetectable, +, weakly expressed, ++, moderately expressed, +++, strongly expressed, NT, not tested.

Sp1 and C/EBPα could bind to each responsive element of the CES1A1 promoter but that Sp1 and C/EBP could not bind to the responsive element of the CES1A2 promoter (Fig. 3).

More recently Fukami *et al.*[73] reported that the sequences of downstream and upstream of the intron of the CES1A2 gene are identical to those of CES1A1 and CES1A3 genes, respectively. A CES1A1 variant, in which exon 1 is converted to that of the CES1A*3* gene (transcript is CES1A2), has recently been identified. It was found that the CES1A2 gene is a variant of the CES1A3 pseudogene (Fig. 3). The expression level of CES1A1 mRNA is much higher than that of CES1A2 mRNA in the liver.[72] Since CES1A1 is highly variable in the individual liver,[74] it was thought that these results provided information on the individual variation of human CES1.

As shown in Table 1, human CES1 and CES2 were highly expressed in the liver and lung, and the small intestine and kidney, respectively. Knowledge of these substrate structure-activity relationships and the tissue distribution of CE isozymes is critical for predicting the pharmacokinetics and pharmacodynamics of pesticides.

6. Possible Role of CE Isozymes in Drug Metabolism

Drug-metabolizing enzymes that are present predominantly in the liver are involved in the biotransformation of both endogenous and exogenous compounds to polar products to facilitate their elimination. These reactions are categorized into phase 1 and phase 2 reactions. CE show ubiquitous tissue expression profiles with the highest levels of CE activity present in liver microsomes in many mammals.[15–17,20,52,74,75–79] CEs are categorized as phase 1 drug-metabolizing enzymes that can hydrolyze a variety of ester-containing drugs and prodrugs. These include angiotensin-converting enzyme (ACE) inhibitors (temocapril, cilazapril, quinapril, and imidapril),[20,38,80,81] anti-tumor drugs (CPT-11 and capecitabin),[37,41,49,82–86] and narcotics (cocaine, heroin and meperidine).[11,87,88] Thus, CEs are one of the most important enzymes involved in prodrug activation, notably with respect to tissue distribution, up-regulation in tumor cells and turnover rates.

We have shown that there are some differences between these families in terms of substrate specificity, tissue distribution, immunological properties, and gene regulation.[27] Analysis of substrate structure versus catalytic efficiency for the ester or carbamate substrates has revealed that a different family of CEs recognizes different structural features of the substrate. For example, the preferential substrates for CES1A1, a human CES1 family isozyme, are thought to be compounds esterified by small alcohols, while those for CES2A1, a human CES2 family isozyme, are thought to be compounds esterified by relatively large alcohols. CES1A1, but not CES2A1, hydrolyzed the methyl ester of cocaine and the ethyl esters of temocapril, meperidine, imidapril and oseltamivir.[20,38,80,89–91]

It was interesting that procainamide inhibited CES1-mediated imidapril hydrolysis.[92] Procainamide is also known as a choline-binding pocket-specific inhibitor[93] and has been reported to competitively inhibit human BuChE.[94] Takai *et al.*[80] reported that a local anesthetic, procaine, and the anticholinergic drug oxybutynin with large alcohol substitutes are substrates for CES2 but not CES1. Procainamide is also a good substrate for CES2. Because the amino acid sequences at the active site were highly conserved among CES1, CES2 and BuChE,[23] it is reasonable to assume that procainamide inhibits CES1-mediated imidapril hydrolysis.

In contrast to the specificity of CES1 for the methyl ester of cocaine, only CES2 hydrolyzed the benzoyl ester of cocaine.[95] The benzoyl esters of cocaine, heroin and CPT-11 (irinotecan) bearing a small acyl moiety and a bulky alcohol group are good substrates for the CES2 isozyme. Irinotecan is one of the most useful anti-tumor drugs. It was interesting that BuChE hydrolyzed the benzoyl ester of cocaine, and also hydrolyzed CPT-11, but not AChE.[96–98] CPT-11 is a relatively potent and selective inhibitor of human AChE that has properties of the acute cholinergic toxicity observed in some patients.[99]

It has been suggested that although these two CE families exhibit broad substrate specificity for ester, carbamate, or amide hydrolysis, these CE isozymes exhibit distinct catalytic efficiencies that correlate with the relative size of the substrate substituents versus that of the enzyme active sites. Tissue-specific expression of CES1 and CES2 was examined by Northern blots, RT-PCR and real-time PCR analysis.

7. Genetic Polymorphism

Geshi *et al.*[81] first reported that a single nucleotide polymorphism (SNP), −816A/C, of the CES1A2 gene is associated with the responsiveness to an angiotensin-converting enzyme (ACE) inhibitor, imidapril, whose activity is achieved by CES1 isozyme. Recently, we re-sequenced the CES1A2 promoter region (~1 kB) in 100 Japanese hypertensive patients. Altogether, ten SNPs and one insertion/deletion (I/D) were identified, among which six SNPs and one I/D residing between −47 and −32 were in almost complete linkage disequilibrium (D′=1.00, r2=0.97).[100] They consisted of a minor and a major haplotype, the allele frequencies of which were 22% and 74%, respectively. The minor haplotype possessed two putative Sp1 binding sites while the major haplotype did not have any Sp1 binding site. The minor haplotype had higher transcription and Sp1 binding activities than the major haplotype *in vitro*.

Later, we studied the relationship between CES1A1 polymorphisms and CES activity in 45 human liver tissues. Namely, six single nucleotide polymorphisms (SNPs), −75G/T, −46A/G, −39A/G, −21C/G, −20G/A, −2G/C and one insertion/deletion (I/D), +71A/del were identified in the promoter region of the CES1A1 gene. The +71 A/del was significantly associated with the conversion efficacy of CPT-11 to SN38 and the level of immunoreactive CES1 protein in the liver microsomes. The +71 A/del was not associated with the CES1A1 mRNA level in the liver, and an *in vitro* reporter assay indicated that +71 A/del does not affect transcription. These results suggest that CES1A1+71 A/del may account, at least in part, for the individual differences in CE activity in human liver microsomes. This polymorphism of CE genes may be a good candidate for studying the pharmacogenetics of the detoxification of drugs and chemicals, including pesticides

8. Novel Biomarker of Organophosphate Exposure

The development of a sensitive biomarker which can detect pesticide poisoning at any stage is very important. In 1981, Kikuchi *et al.*[101] reported that rat plasma β-glucuronidase (BG) activity was increased 2 h post-treatment with organophosphorous pesticides (OPs). Subsequently, it was reported that a complex of BG and egasyn, which is an accessory protein of BG, exists in the liver microsomal membrane. Egasyn was found to be a CEs isozyme.[24,59] BG is loosely bound to egasyn, and the complex is easily dissociated by OP exposure. Subsequently, several studies reported that intake of OP by the liver causes the release of BG into plasma.[102] Fujikawa *et al.*[103] reported that a single administration of Ops, including *O*-ethyl *O*-4-nitrophenyl phenylphosphonothioate (EPN), acephate and chlorpyrifos and bis(p-nitrophenyl)phosphate (BNPP) as a non-OP to rats led to a 100-fold increase in plasma BG activity over the control. In human studies, Inayat-Hussain *et al.*[104] reported that plasma BG activity in a group of chronically OP-exposed farmers was significantly increased compared to that in non-OP-exposed controls. In these cases, no significant difference in BuChE activity was detected between the farmers and control groups. Recently, Soltaninejad *et al.*[105] observed a significant increase in blood BG activity in patients severely intoxicated by OP exposure compared to controls. In 2010, Ueyama *et al.*[106] reported that 42 male adults were classified into two groups; the first group consisted of 21 pesticide control operators (PCOs) who had not sprayed OPs within 3 days prior to their health check (PCO1), while the second group was composed of 21 PCOs who had sprayed OP insecticides within 3 days prior to their health check (PCO2). According to the monitoring data, plasma BG activity in the PCO2 group was higher than in PCO1. In these cases, no significant decrease in BuChE was observed.

These findings suggested that blood BG can be a more sensitive biomarker of OP exposure than the inhibition of AChE and BuChE activities in humans. We concluded that the cross-sectional studies in this paper are useful for monitoring OP exposure in a population of pest control operators

9. Conclusions and Future Directions

Multiple CEs play an important role in the hydrolytic biotransformation of a vast number of structurally diverse drugs. These enzymes are major determinants of the pharmacokinetic behavior of most therapeutic agents containing an ester or amide bond. Several factors influence CE activity, either directly or at the level of enzyme regulation. In the clinical field, drug elimination is decreased and the incidence of drug-drug interactions increases when two or more drugs compete for hydrolysis by the same CE isozyme.

Exposure to chemicals or lipophilic drugs can result in the induction of CE activity. Several drug-metabolizing enzymes, such as cytochrome P450, UDPGT-glucuronosyltransferase and sulfotransferase have been extensively studied to clarify substrate specificity using molecular cloning and cell expression systems. Consequently, the novel findings obtained reveal that the substrate specificity of CE is, at least in part, explained by differences in the nucleotide sequences of the individual CE isozymes.

It is clear that membrane-bound-type CE isozymes in microsomes are required to possess the KDEL tetrapeptide motif at the carboxy terminal of the molecule. Mammalian CEs have been found to have acyl glycerol, acyl-CoA, and acylcarnitine hydrolyzing activities *in vitro*; however, the physiological roles of CE remain unclear. To clarify the substrate specificity of each CE isozyme, we have begun to search for the substrate recognition site of each isozyme.

In the present review, we described the substrate specificity and tissue-specific expression profile of CE isozymes; therefore, the successful design of ester-containing drugs will be greatly improved by further detailed analysis of the mechanism of action and substrate recognition sites of CE isozymes

in mammals.

In conclusion, the molecular-based information on CEs in this review is useful to understand the multiplicity and substrate specificity of the CE family associated with the efficacy, side effects and toxicity of chemicals.

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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. © Pesticide Science Society of Japan

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.

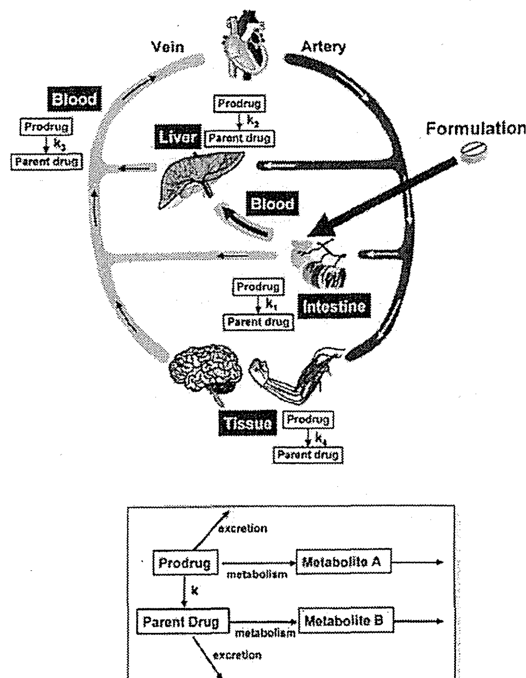

Fig. 1. Distribution of prodrug after oral administration and bioconversion of prodrug.

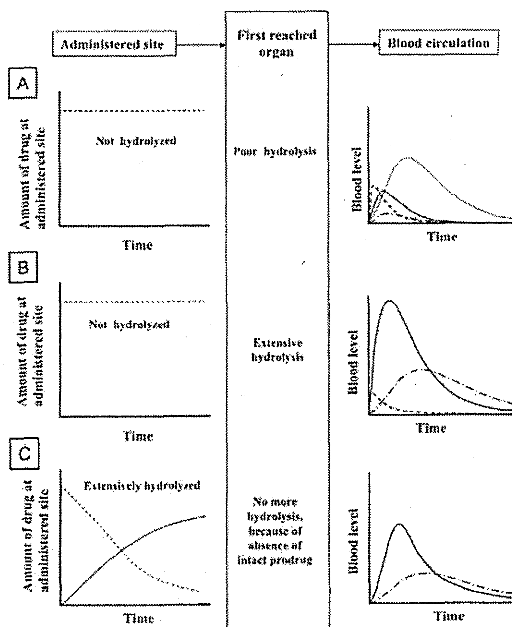

Fig. 2. Drug amount at administered site and blood after administration of prodrug. When prodrug is orally administered, the intestine is the administered site and the first organ reached is the liver. In cutaneous and intravenous administration, the administered site is the skin and veins, respectively, 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.

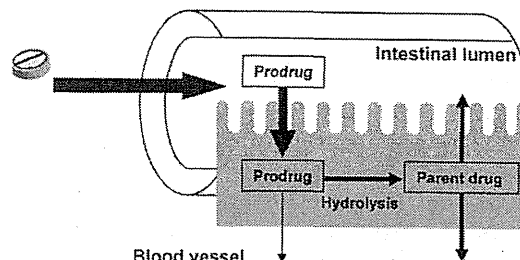

Fig. 3. Absorption and hydrolysis of prodrug in the intestinal epithelial cell.

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-

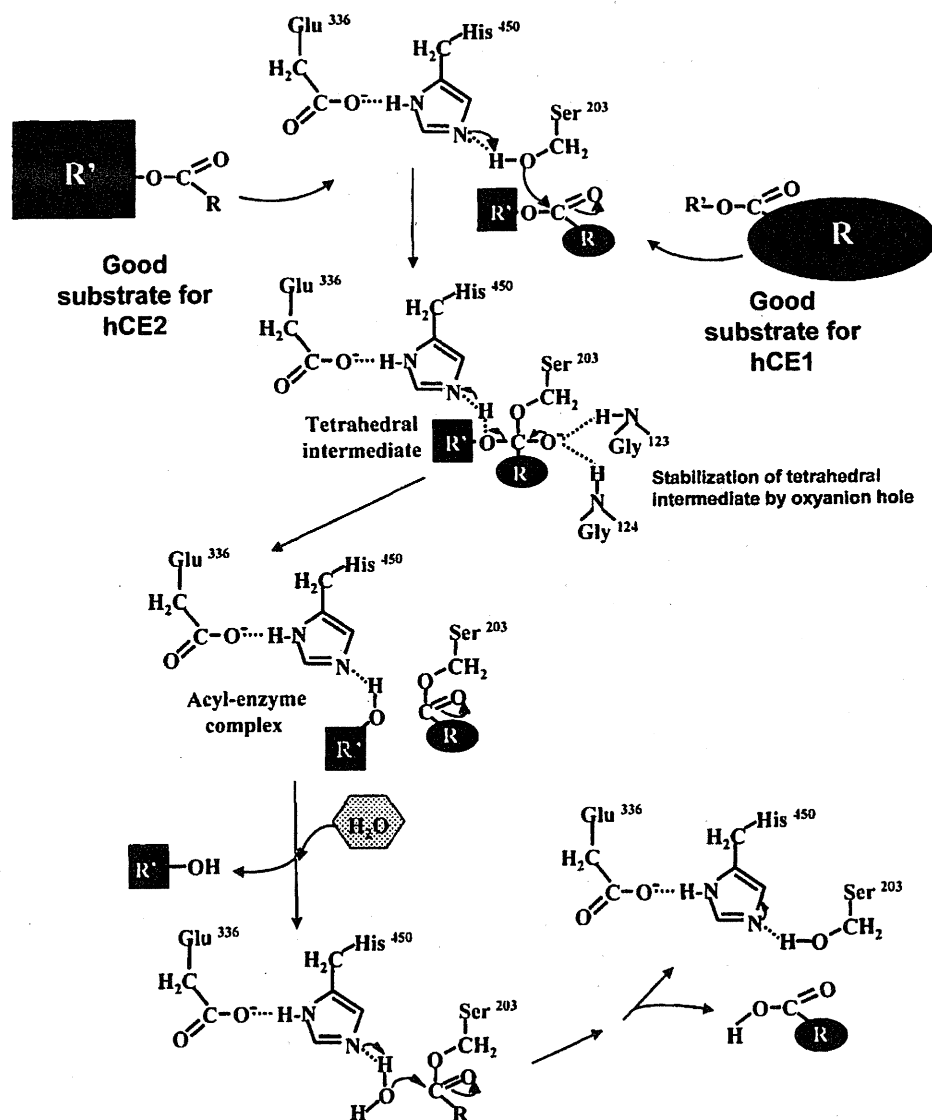

Fig. 4. Hydrolyzing mechanism of CES.

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

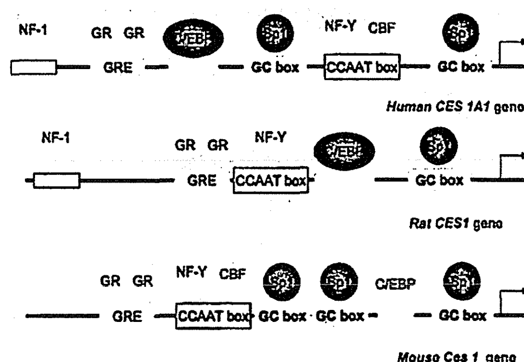

Fig. 5. Structure of the 5′ flanking region of mammal and human CES1 genes.