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Sensing viral invasion by RIG-I like receptors

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Cellular responses to pathogen invasion are crucial for maintaining cell homeostasis and survival. The interferon (IFN) system is one of the most effective cellular responses to viral intrusion in mammals. Viral recognition by innate immune sensors activates the antiviral IFN system. Retinoic acid-inducible gene I (RIG-I) like receptors (RLRs) are DExD/H box RNA helicases that sense viral invasion. RLRs recognize cytoplasmic viral RNAs and trigger antiviral responses, resulting in production of type I IFN and inflammatory cytokines. Unique and common sensing mechanisms among RLRs have been reported. In this review, recent progress in the understanding of antiviral responses by RLRs is summarized and discussed.

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Introduction

Virus infection induces numerous host responses to eliminate pathogen invasion. Host antiviral responses are operated by germline-encoded cellular receptors that recognize specific patterns of foreign molecules, termed pathogen-associated molecular patterns (PAMPs). PAMPs are detected by the 'sensor' molecules, known as pattern recognition receptors (PRRs), which play a crucial role in triggering host innate immunity, a primary cellular defense system. Recent studies have identified several innate immune receptors including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and RLRs, and clarified their roles in antiviral signaling by sensing virus-derived molecules.

In this review, we focus on RLRs, which detect cytoplasmic viral RNA PAMPs. For the function of TLRs and NLRs, refer to reviews published elsewhere [1,2].

RLR-mediated signaling

RLRs are Asp-Glu-Ala-Asp (DEAD) box containing RNA helicases. To date, three members including RIG-I, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) have been identified. All RLRs share helicase domain and C-terminal domain (CTD), however LGP2 lacks a caspase activation and recruitment domain (CARD), that is critical for signal transduction; thus, LGP2 has been suggested as the regulatory molecule for RIG-I and MDA5 [3,4].

Upon viral infection, RLRs recognize PAMP RNAs (Table 1) and undergo conformational change mediated by ATPase/Helicase activity, which results in the exposure of CARD [5,6]. Although RIG-I and MDA5 sense distinct viral infections [7] (Table 2), they share a common mitochondria-localized downstream signal adaptor, interferon promoter stimulator-1 (IPS-1, also termed MAVS, VISA or Cardif) for signal transduction [8]. Upon interaction between RLR and IPS-1 via their CARD-CARD association, IPS-1 conforms aggregates which share several features of prions [9]. The mitochondrial component, mitofusin 1 (MFN1) plays a critical role in the IPS-1 aggregation by regulation of the mitochondrial fusion and fission [10]. This assembly further results in the recruitment of several ubiquitin ligases (TRAFs) and kinase complexes (TBK1 or IKKε and IKKα/β/γ complex). A recent study suggested that GEF-H1, a microtubule network-associated protein, regulates the trafficking of IFN signaling by mediating the interaction between TBK1/IKKε and IRF3 [11]. Eventually, the activation of transcription factors IRF3 and IRF7, as well as NF-κB, leads to the production of type I IFNs and pro-inflammatory cytokines [8] (Figure 1).

The produced IFN becomes a messenger of the 'warning sign' for both IFN-producing and bystander cells, and promotes the 'second round' antiviral responses by inducing the expression of hundreds of interferon-stimulated genes (ISGs). The antiviral roles of several representative ISGs, such as PKR, OASs and RNase L, are well-characterized. Recent studies further elucidated the function of another ISG, IFITs, in their antiviral activity. IFITs, especially IFIT1, IFIT2 and IFIT5, recognize 5′-triphosphorylated (5′ppp) RNA or capped RNA lacking 2′-O-methyl groups and sequester such RNA from translation, leading to the inhibition of viral replication [12•,13••].

RNA ligands of RIG-I

Does RIG-I recognize RNA with 5′-triphosphate?

In 2006, two groups simultaneously provided significant evidence that triphosphate moiety on the 5′ end is an

Table 1

RLR agonists

RNA types	RNAs/viruses	Characteristics	Sensor	References
Artificial RNA	5′ppp copy-back RNA	In vitro transcribed RNA	RIG-I	[18,19]
	5′ppp AU-rich RNA	Pol III product from poly dA:dT	RIG-I	[26,27]
	poly I:C short	Short dsRNA (<1 kb)	RIG-I	[24]
	poly I:C long	Long dsRNA (>7 kb)	MDA5	[24]
Natural RNA	IAV genomic RNA	5′ppp with panhandle structure	RIG-I	[17]
	HCV 3′UTR U/UC rich RNA	5′ppp dependent	RIG-I	[25]
	IAV 3′UTR U/A rich RNA	5′ppp independent	RIG-I	[62]
	SeV, VSV DI RNA	5′ppp copy-back dsRNA	RIG-I	[21,39••,63]
	VV, EMCV	High molecular weight RNA	MDA5	[34]
	CVB3, Mengo	Replication intermediates	MDA5	[33]
	PIV5 L mRNA	RNase L product	MDA5	[35]
	EMCV L antisense	LGP2-interacting RNA	MDA5/LGP2	[64]
Host RNA	5′OH-3′p short RNA	RNase L product	RIG-I/MDA5	[28]

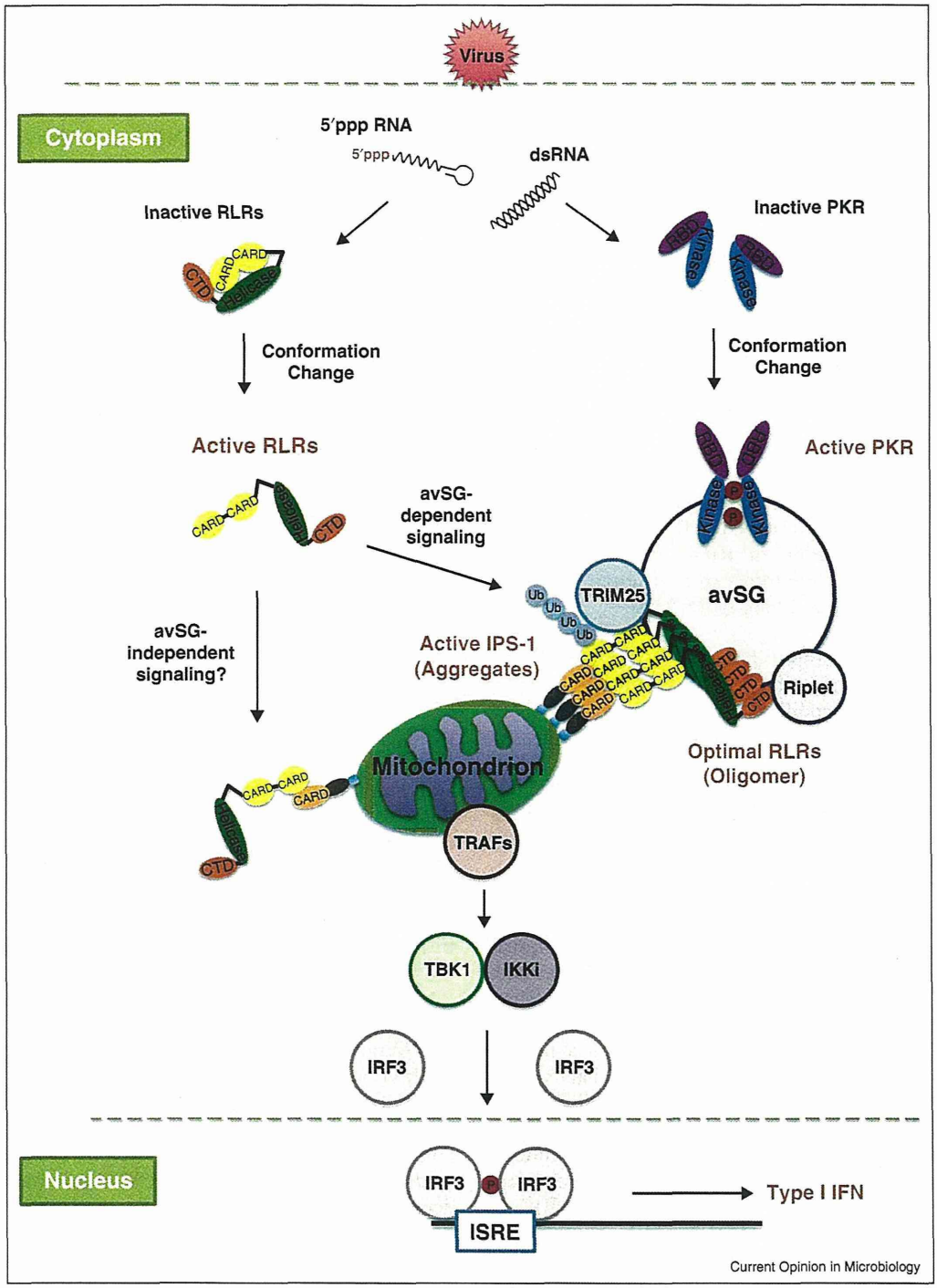
essential determinant of RIG-I activation using single-stranded (ss) '*in vitro* transcribed' RNA (ivtRNA) or a viral genome containing 5′ppp [14–16]. Rehwinkel *et al.* also proved that viral genomes bearing 5′ppp trigger IFN responses [17]. However, it became clearer in the following two studies that 5′ppp itself is not sufficient for efficient RIG-I activation [18,19]. These reports commonly found that, unlike 5′ppp ivtRNA, chemically synthesized 5′ppp ssRNA did not activate RIG-I and they also realized that products of *in vitro* transcription by phage RNA polymerase retained the unexpected 'copy-back' structure. Therefore they concluded that an additional double-stranded region or a stem-loop structure is necessary for a true RIG-I agonist. Indeed, it was clarified that Sendai virus (SeV) and vesicular stomatitis virus (VSV) produce RIG-I agonist RNA, known as a 'defective-interfering (DI) RNA', which contains a copy-back structure with 5′ppp [20,21]. Moreover, recent studies showed that PAMP RNA from negative-strand ssRNA viruses, including influenza A, rabies,

Table 2

List of viruses and their known sensors

Sensor	Viruses	Families/genome	References
RIG-I	Newcastle disease virus	Paramyxoviridae/(−) ssRNA, non-segmented	[7,22]
	Sendai virus		[7]
	Respiratory syncytical virus		[32]
	Vesicular stomatitis virus	Rhabdoviridae/(−) ssRNA, non-segmented	[3,7]
	Rabies virus		[15]
	Influenza A virus	Orthomyxoviridae/(−) ssRNA, segmented	[7]
	Influenza B virus		[32]
	Rift Valley fever virus	Bunyaviridae/(−) ssRNA, segmented	[65]
	La Crosse virus		[65]
	Hepatitis C virus	Flaviviridae/(+) ssRNA, non-segmented	[25]
	Japanese encephalitis virus		[7]
	Epstein–Barr virus	Gammaherpesviridae/dsDNA	[66]
MDA5	Encephalomyocarditis virus	Picornaviridae/(+) ssRNA, non-segmented	[7]
	Theiler's murine encephalitis virus		[7]
	Mengovirus		[7]
	Coxackie virus		[33]
	Entero virus		[33]
	Human parechovirus		[33]
	Equine rhinitis A virus		[33]
	Saffold virus		[33]
	Norovirus	Caliciviridae/(+) ssRNA, non-segmented	[30]
	Vaccinia virus	Poxviridae/dsDNA	[34]
RIG-I/MDA5	Westnile virus	Flaviviridae/(+) ssRNA, non-segmented	[32]
	Dengue viurs		[32]
	Measles virus	Paramyxoviridae/(−) ssRNA, non-segmented	[67]
	Semliki forest virus	Togaviridae/(+) ssRNA, non-segmented	[31]

Figure 1



RLR signal pathway. Upon viral infection, non-self RNAs, such as 5′ppp-containing structured RNA or dsRNA, are recognized by host cytoplasmic sensors, RLRs and PKR. Recognition of RNA ligands induces conformational changes of RLRs and PKR, leading to the initiation of downstream signaling cascades. Activated PKR immediately induces avSG, where antiviral proteins and signal molecules interact, and augments RLR signaling. E3 ubiquitin ligases-mediated CARD-CARD interaction between RLRs and IPS-1 recruits TRAFs and kinase complexes (TBK1 & IKKε) to facilitate IRF3 phosphorylation and nuclear translocation, resulting in the expression of type I IFN genes.

and measles virus, possess conserved panhandle structures with 5′ppp in their genomes, further confirming that 5′ppp along with secondary structures is an indispensable characteristic of RIG-I activators.

dsRNA activates RLRs

dsRNA is a classical non-self RNA, which is not produced in uninfected cells due to a lack of RNA-dependent RNA polymerase in mammalian cells. Both RIG-I and MDA5 appear to regulate the induction of type I IFN after stimulation by dsRNA [22]. Since artificial dsRNA, polyinosinic:polycytidylic acid (poly I:C), does not possess 5′-triphosphate, several studies using various 5′-modified or 3′-modified dsRNAs also showed that 5′ppp is dispensable for RIG-I activation [5,23]; it is generally assumed that recognition of dsRNA by RIG-I does not require 5′ppp. Interestingly, it was discovered that the length of dsRNA is a critical determinant that enables RNA ligands to turn on either RIG-I or MDA5 activation [24]. It was revealed that short dsRNA (<1 kb) elicited IFN production through RIG-I while long dsRNA (>7 kb) failed to activate RIG-I, but efficiently activate MDA5. Related to the size-dependent activation of RIG-I by dsRNA, it is suggested that RIG-I conforms to a different structure when bound to short and long dsRNA [5].

The dsRNA chain length-dependent recognition was confirmed by viral infection [24]. For example, viruses that produce undetectable (Influenza A virus) or short dsRNA (VSV) activate RIG-I-dependent signaling. In contrast, long dsRNA-producing viruses, such as encephalomyocarditis virus (EMCV) induce IFN signaling through MDA5. Interestingly, reovirus, whose genome is composed of different-sized segmented dsRNA, is recognized by both RIG-I and MDA5 [24].

RNA sequence specificity

Although sensing of dsRNA does not seem to require sequence specificity, the RNA sequence is an important feature for the recognition of 5′ppp RNAs by RIG-I. Saito *et al.* found that 3′-untranslated region (3′UTR) of the hepatitis C virus (HCV) genome (ssRNA) is a potent RIG-I activator [25]. On screening of the RIG-I activating domain by dissecting the HCV 3′UTR, they discovered that the uridine-rich region has the potential to trigger IFN signaling through RIG-I in a 5′ppp dependent manner. Nucleotide substitution analysis further revealed that adenosine-rich RNA also potentially activates RIG-I. Moreover, the authors also showed that IFN induction by the full-length HCV genome is dependent on 3′UTR *in vivo*, confirming that the uridine-rich sequence is a crucial PAMP for the recognition of HCV by RIG-I.

PAMP production by host enzymes

In 2009, two groups simultaneously reported that AT-rich dsDNA triggers IFN signaling through the RIG-I pathway [26,27]. It was discovered that AT-rich, but not GC-rich or IC-rich dsDNA, is a template for RNA polymerase III that produces 5′ppp-containing AU-rich RNA. Since transcription may occur for both strands of a template, RNA transcripts are perfectly complementary, producing 5′ppp-dsRNA.

By definition, 'non-self RNAs' are derived from a pathogen, however, one report suggests the conversion of 'self RNAs' into immune-stimulatory molecules. Malathi *et al.* discovered that small RNAs produced from the cleavage of both viral and host RNA by RNase L can activate interferon signaling through both RIG-I and MDA5 [28]. Cleaved products are small RNAs (<200 nt) with 5′-hydroxyl (5′-OH) and 3′-monophosphoryl (3′-P) groups. Interestingly, loss of 3′-monophosphate abolished its ability to activate RLR, suggesting a 3′-monophosphate-dependent sensing mechanism for RLR signaling. The authors further suggested the requirement of a higher order structure in addition to 3′-P for this regulation [29].

RNA ligands of MDA5

MDA5 recognizes long dsRNA

Compare to RIG-I ligands, very little is known about the biochemical specificities of 'MDA5-activating' RNA ligands. To date, several approaches have elucidated that MDA5 mainly senses positive single-stranded RNA or dsRNA viruses, such as *Picornaviridae*, *Caliciviridae*, *Togaviridae*, *Flaviviridae*, and *Reoviridae*. While IFN induction by *Picornaviridae* and *Caliciviridae* exclusively detected by MDA5 [7,30], MDA5 is partially involved in antiviral responses induced by *Togaviridae* [31], *Flaviviridae* [32] and *Reoviridae* [24]. These viruses produce or possess 'long dsRNA' and this is consistent with the central concept that MDA5 senses long dsRNA. Recently, Feng *et al.* suggested that MDA5 senses neither genomic RNA nor viral mRNA, but recognizes 'replication intermediates' generated during minus-strand RNA synthesis [33], further supporting the idea that long dsRNA is a critical determinant of an MDA5 ligands.

RNA web

In addition to length dependency, there is another hallmark of MDA5 ligand in relation to the RNA structure. Recently, Pichlmair *et al.* suggested the new concept that activation of MDA5 requires a 'high-order' RNA structure [34]. Interestingly, 'pure' long dsRNA (>10 kb) generated by the viruses such as EMCV or vaccinia virus (VV) does not trigger IFN expression even though MDA5 interacts with it. However, 'high molecular weight' viral RNA that contains both ssRNA and dsRNA, robustly induces innate immune responses. Since MDA5 recognition of these RNAs does not require sequence specificity, they concluded that MDA5 activation requires an 'RNA web' rather than just 'simply long' dsRNA. Since this result was confirmed with natural viral RNA, their finding may provide a significant clue for the identification of natural MDA5 ligand.

Cooperation of RNase L for MDA5 sensing

Interestingly, there is a recent report showing that MDA5 can recognize viral mRNA and induce IFN signaling. Luthra *et al.* showed that parainfluenza virus 5 (PIV5) mRNA coding L protein can be converted to a potent MDA5 ligand by RNase L [35]. It is of interest to note that only a specific region of viral mRNA cleaved by RNase L activates MDA5, suggesting that MDA5 requires a specific RNA sequence or structure for recognition.

Contribution of LGP2 to RLR sensing

Compared to other RLRs, the functional role of LGP2 in viral recognition is still not well understood. LGP2 was originally thought to function as a negative regulator of RLR signaling due to the lack of the signaling domain, CARDs [3]. Indeed, several earlier reports showed the negative regulation of RLR signaling by overexpression of LGP2 [3,36]. However, two recent independent studies using a 'LGP2-knockout' system suggested a positive role of LGP2 in RLR sensing [4,37]. These two studies confirmed impaired IFN production in LGP2-deficient cells by picornavirus, indicating that LGP2 functions positively in MDA5-derived IFN signaling. However, the functional role of LGP2 in RIG-I-induced antiviral responses is inconsistent with these studies; thus, the contribution of LGP2 to sensing by RIG-I is controversial. It is proposed that LGP2 may cooperate with either MDA5 or RIG-I to transmit a signal to IPS-1 through their CARDs. Interestingly, LGP2 is dispensable for IFN production by synthetic RNA molecules such as poly I:C and 5′ppp RNA. Although ATPase activity of LGP2 is essential for this regulation [4], the exact mechanism of LGP2-mediated augmentation remains to be clarified.

Post-translational modification: the mainspring of RLR signaling

Upon viral infection, antiviral IFN signaling is immediately propagated through the cooperative association of multiple antiviral molecules. During the signaling cascade, antiviral proteins undergo various biochemical or physical modifications, such as oligomerization, ubiquitination and phosphorylation.

It was reported that RIG-I and MDA5 are oligomerized upon ligand recognition. Indeed, artificial oligomerization of RIG-I using a chemical cross linker can activate an antiviral response without a virus or dsRNA stimulus [38]. Recent studies suggested that oligomerization of RIG-I occurs through its ATPase activity and is required for optimal RIG-I activation [39••,40•]. Peisley *et al.* showed that RIG-I binds to the end region of dsRNA without ATP hydrolysis but upon ATP treatment, RIG-I forms a 'filament-like' oligomer along dsRNA [40•]. Unlike RIG-I, however, MDA5 is capable of assembling a filament in an ATP-independent manner, suggesting distinct sensing mechanisms between RIG-I and MDA5 [41•]. In addition, Jiang *et al.* reported that lysine-63 (K63) poly-ubiquitination on the CARD domain induces a hetero-tetrameric complex of RIG-I and further elucidated that ubiquitin-induced RLR oligomerization is critical for downstream signal transduction [42••]. Thus, oligomerization of RLR is a significant hallmark of RLR activation.

The importance of K63 poly-ubiquitination on RIG-I by ubiquitin ligases TRIM25 and Riplet has been previously reported [43,44]. Indeed, multiple ubiquitin ligases are involved in the regulation of innate immune signaling [45]. Upon K63 ubiquitination-mediated RIG-I/IPS-1 interaction, IPS-1 forms a fibril-like structure that converts 'normal' IPS-1 to 'functional' IPS-1, thus termed 'prion-like-aggregates', leading to the propagation of antiviral signaling [9]. Thus, ubiquitination seems to be a critical post-translational modification for governing the antiviral signaling from 'RLR activation' to 'signal transduction'.

Phosphorylation is one of the best-studied post-translational modifications that switch many cellular signaling pathways on and off. Indeed, several kinases are involved in IFN signaling and play crucial roles in host antiviral responses. dsRNA-dependent protein kinase (PKR) is a classic IFN-inducible antiviral protein that comprises a dsRNA-binding motif and a kinase domain [46]. Recently, it was suggested that PKR is a key factor for the induction of cytoplasmic bodies called 'stress granule' (SG) by viral infection and we further demonstrated that SGs provide a critical platform for interactions between antiviral proteins, including RLRs, DHX36, RNase L and OAS1, and non-self RNA ligands, thus termed 'antiviral stress granule' (avSG) [47••,48–50] (Figure 1) More recently, it was shown that the ubiquitin ligases TRIM25 and Riplet are co-localized with SG after viral infection or poly I:C transfection, further suggesting the significant role of avSG in RLR signal transduction [44,50] (Figure 1).

Viral evasion of RLR sensing

The loser of the battle between a virus and host will face a 'dead-end', thus arming it with a better strategy that is essential for survival. Recent reports showed multiple strategies of viruses to evade RLR sensing by encoding suppressors that target signaling molecules in antiviral responses.

Several viral proteins directly target RLRs. For example, influenza virus NS1, a multifunctional antagonist of host immune responses [51], inhibits ubiquitination of RIG-I CARD by interrupting TRIM25 [52]. In addition, hijacking of RIG-I and TRIM25 by viral protein abrogates IFN signaling [53]. Moreover, it is known that V protein of *paramyxoviridae* member viruses directly binds to MDA5 and suppresses the function of MDA5 [54]. Critical signal

adaptor, IPS-1 is also an attractive target of HCV that induce cleavage of IPS-1 by viral protease, NS3/4A [55]. Furthermore, function of downstream signal molecules such as TBK1 and IRF3 is also hampered by viral infection [56–58].

In addition to direct inhibition of the signaling molecules, several viruses also counteract IFN production by preventing or disrupting the formation of avSG. It is reported that influenza viral protein NS1 inhibits PKR activation to prevent avSG formation [47••,59]. EMCV also blocks antiviral signaling by disrupting avSG through cleavage of G3BP by viral protease 3C [48]. Furthermore, several viruses antagonize the induction of avSG formation by preventing dsRNA accumulation that would otherwise induces antiviral signaling [60,61].

Conclusions

It has been a decade since the 'long-sought' cytoplasmic viral sensors, RLRs, were identified. During the last decade, numerous efforts by colleagues in this field have advanced our understanding of the cytoplasmic antiviral sensing system. However, there are still unsolved fundamental questions that remain to be addressed.

Although accumulated research has identified the essential properties of RLR-activating ligands, our knowledge is limited due to the physiological differences between 'artificial' and 'natural' RLR agonists. Therefore, it is important to investigate, firstly, what types of RNA are produced by different viral infections, secondly, which RNA molecules from viral replication are truly responsible for RLR activation, thirdly, what is the exact molecular feature of RLR-activating viral RNAs and finally, how each virus evades host immune responses. By understanding these issues, it may be possible to develop 'order-made' antiviral therapeutics or vaccines for 'viral strain-specific' clinical treatment.

Another important issue to be considered is that, although major players in IFN signaling have been identified, the molecular mechanisms of signal transduction and termination are still poorly understood. Since autoimmune disorders are often related to the loss of 'self-immune control', understanding the precise molecular mechanisms of the signal transduction may enable us to target specific signal molecules to relieve 'hypersensitive' immune reactions.

Finally, it would be also worth connecting RLRs to other applications, for instance, developing anti-cancer or anti-aging treatments for the next decade.

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Short Communication

Validation of uPA/SCID Mouse with Humanized Liver as a Human Liver Model: Protein Quantification of Transporters, Cytochromes P450, and UDP-Glucuronosyltransferases by LC-MS/MS[S]

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ABSTRACT

Chimeric mice with humanized liver (PXB mice) have been generated by transplantation of urokinase-type plasminogen activator/severe combined immunodeficiency mice with human hepatocytes. The purpose of the present study was to clarify the protein expression levels of metabolizing enzymes and transporters in humanized liver of PXB mice transplanted with hepatocytes from three different donors, and to compare their protein expressions with those of human livers to validate this human liver model. The protein expression levels of metabolizing enzymes and transporters were quantified in microsomal fraction and plasma membrane fraction, respectively, by means of liquid chromatography–tandem mass spectrometry. Protein expression levels of 12 human P450 enzymes, two human UDP-glucuronosyltransferases, eight human ATP binding cassette (ABC) transporters, and eight human solute carrier transporters were determined. The variances of protein expression levels among samples from mice humanized with hepatocytes from all donors were significantly greater than those from samples obtained from mice derived from each individual donor. Compared with the protein expression levels in human livers, all of the quantified metabolizing enzymes and transporters were within a range of 4-fold difference, except for CYP2A6, CYP4A11, bile salt export pump (BSEP), and multidrug resistance protein 3 (MDR3), which showed 4- to 5-fold differences between PXB mouse and human livers. The present study indicates that humanized liver of PXB mice is a useful model of human liver from the viewpoint of protein expression of metabolizing enzymes and transporters, but the results are influenced by the characteristics of the human hepatocyte donor.

Introduction

Species differences in drug metabolism and transport in the liver between humans and experimental animals are a critical issue during drug development. To overcome this problem, chimeric mice with humanized liver (PXB mice; PhoenixBio Co., Ltd., Hiroshima, Japan) have been generated by transplantation of human hepatocytes into albumin enhancer/promoter-driven urokinase-type plasminogen activator/severe combined immunodeficiency (uPA$^{+/+}$/SCID) mice; in these mice approximately 80% of the hepatocytes are human (Tateno et al., 2004). PXB mice generate human-specific metabolites (Inoue et al., 2009; Kamimura et al., 2010; Yamazaki et al., 2010; De Serres et al., 2011), and pregnane X receptor (PXR)–dependent induction of metabolizing enzymes was observed when the mice were treated with a human PXR ligand (Hasegawa et al., 2012). Therefore, the liver of PXB mice is considered to be potentially useful as a model of human liver for studies of drug metabolism.

The uptake of most drugs from circulating blood into the liver at the sinusoidal membrane of hepatocytes involves active transport. The drugs subsequently undergo biotransformation by intracellular enzymes such as cytochrome P450 (P450) and UDP-glucuronosyltransferase (UGT), and the parent drug or its metabolites are eventually excreted from the hepatocytes by canalicular and/or sinusoidal transporter proteins. Therefore, expression analyses of metabolic enzymes and transporters in the liver of PXB mice are essential to validate the model. For example, it has been established by means of quantitative PCR and quantitative immunoblot analyses that PXB mice with a high replacement ratio express eight human P450s and human phase II enzymes, including three UGTs, at levels similar to those in human liver (Katoh et al., 2004,2005). Gene expression of the human ATP binding cassette (ABC) transporters and human solute carrier (SLC) transporters was also confirmed in humanized liver (Nishimura et al., 2005; Kikuchi et al., 2010). However, protein expression of drug transporters has not yet been quantitatively analyzed in PXB mice. This is important, because we recently showed that there is a poor correlation between protein and mRNA expression levels of metabolizing enzymes (except

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ABBREVIATIONS: ABC, ATP binding cassette; BCRP, breast cancer resistance protein; BSEP, bile salt export pump; CV, coefficient of variance; γ-GTP, γ-glutamyl transpeptidase; LC-MS/MS, liquid chromatography–tandem mass spectrometry; LLOQ, lower than the limit of quantification; MRM, multiplexed multiple reaction monitoring; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PXB mice, chimeric mice with humanized liver; P450R, NADPH-cytochrome P450 reductase; UGT, UDP-glucuronosyltransferases; uPA/SCID, urokinase-type plasminogen activator/severe combined immunodeficiency.

CYP3A4) and transporters in human livers; for example, the correlation coefficients (r^2) were less than 0.3 for CYP1A2, 2C9, 2A6, 2E1, UGT1A1, CYP2B7, multidrug resistance-associated protein 2 (MRP2), multidrug resistance protein 1 (MDR1), bile salt export pump (BSEP), multidrug and toxin extrusion protein 1 (MATE1), organic cation transporter (OCT1), sodium/taurocholate cotransporting polypeptide (NTCP), and organic anion transporting polypeptide (OATP)1B3 (Ohtsuki et al., 2012). Furthermore, metabolizing activities of P450s such as CYP2C9, 2C19, 2D6, and 2E1 were correlated to expression levels of protein rather than mRNA (Ohtsuki et al., 2012). In addition, human hepatocytes are transplanted to produce the PXB mice, so it is also important to consider the influence of the donor on the protein expression of metabolizing enzymes and transporters.

We have recently developed a liquid chromatography–tandem mass spectrometry (LC-MS/MS)–based protein quantification method that does not require antibodies (Kamiie et al., 2008). In this method, the target protein concentration in a sample is determined after enzymatic digestion by quantifying one or more peptide fragments specific to the target molecule. By using this method, we have measured protein expression levels of metabolizing enzymes and transporters in human and mouse livers (Kamiie et al., 2008; Kawakami et al., 2011; Ohtsuki et al., 2012). Since the target peptide is identified by mass-weight information, a single amino acid difference can be distinguished. Furthermore, the specificity, accuracy, and dynamic range of quantification by LC-MS/MS–based analysis [coefficient of variance (CV) < 20% and three-orders-of-magnitude dynamic range] are greatly superior to those in the case of immunoblot analysis (Kamiie et al., 2008; Kawakami et al., 2011; Ohtsuki et al., 2011). Therefore, this method was considered suitable for validating PXB mouse as a human liver model in terms of protein levels in the liver.

The purpose of the present study was to clarify the protein expression levels of metabolizing enzymes and transporters in liver of PXB mice transplanted with human hepatocytes from different donors by using LC-MS/MS, and to compare the protein expression levels with those of human livers to validate PXB mouse as a model of human liver.

Materials and Methods

Generation of PXB Mice. The present study was approved by the Ethics Committees of the Graduate School of Pharmaceutical Sciences, Tohoku University, and PhoenixBio Co., Ltd. The experiments in this report conformed to the guidelines established by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University, and PhoenixBio Co., Ltd. The cryopreserved human hepatocytes from donor BD85 (black, male, 5 years old), BD72 (white, female, 10 years old), and BD87 (white, male, 2 years old) were purchased from BD BioSciences (San Jose, CA) (Supplemental Table 1). The chimeric mice with humanized liver were generated by the method described previously (Tateno et al., 2004). Briefly, uPA$^{+/+}$/SCID mice were prepared (Tateno et al., 2004), and at 3 weeks after birth they were injected with human hepatocytes through a small left-flank incision into the inferior splenic pole. The concentration of human albumin in the blood of the chimeric mice and the replacement index (RI; the rate of the replacement from mouse to human hepatocytes) were measured using latex agglutination immunonephelometry (LX Reagent "Eiken" Alb II; Eiken Chemical, Tokyo, Japan) and anti-human specific cytokeratin 8 and 18 antibody (Cappel Laboratory, Cochranville, PA), respectively (Supplemental Table 1). There was a good correlation between the human albumin concentration and the RI (Tateno et al., 2004). In this study, the chimeric mice used were 13–14 weeks of age.

LC-MS/MS–Based Protein Quantification Analysis. Microsomal and plasma membrane fractions of liver were prepared as described previously (Ohtsuki et al., 2012). For details, see Supplemental Data. Protein quantitation of the target molecules was simultaneously performed by means of high-performance (HP)LC-MS/MS for metabolizing enzymes or nanoLC-MS/MS for transporters with multiplexed multiple reaction monitoring (multiplexed MRM) as described previously (Ohtsuki et al., 2011; Shawahna et al., 2011; Uchida et al., 2011). Protein expression levels were determined by quantifying specific target peptides produced by trypsin digestion (Supplemental Table 2). Absolute amounts of each target peptide were determined by using an internal standard peptide, which is a stable isotope-labeled peptide with an amino acid sequence identical to that of the corresponding target peptide. Details of the quantification procedure are given in Supplemental Data.

One specific peptide was selected for quantification of each target protein and measured at four different MRM transitions. The amount of each peptide was determined as an average of three or four MRM transitions from one sample. In cases where signal peaks of fewer than three transitions were obtained, the amount of peptide in the sample was defined as under the limit of quantification. The absolute expression amount of CYP3A4 was calculated from the quantitative data obtained for a peptide generated from both CYP3A4 and CYP3A43 by subtracting the value obtained for a peptide that is specific for CYP3A43. Since CYP3A43 was under the limit of quantification in all samples, quantification values obtained with CYP3A4/43 peptides were used as those of CYP3A4.

For the comparison of protein expression levels between humanized liver of PXB mice and human liver, the data for microsomal fraction of 17 human liver biopsies were taken from our previous publication (Ohtsuki et al., 2012).

Statistical Analysis. Statistical significance of differences among donors was determined by one-way analysis of variance followed by the Bonferroni test using Origin 9 software (OriginLab Corp., Northampton, MA).

Results

Protein Expression Levels of Metabolizing Enzymes in Microsomal Fraction of PXB Mouse Liver. Protein expression levels of 12 human P450 enzymes, human NADPH-cytochrome P450 reductase (P450R), Na^+/K^+ ATPase, and γ-glutamyl transpeptidase (γ-GTP) were determined in liver microsomal fraction of PXB mice with transplanted hepatocytes from three different donors (Table 1). Two human UGT enzymes were determined in hepatocytes from two different donors. Na^+/K^+ ATPase and γ-GTP are membrane markers, and both the human and mouse molecules were quantified. The coefficients of variance of their quantification values were 14.5% and 14.1%, respectively, among 11 samples, and the values were not significantly different among the three donors. This suggests that the purity of the microsomal fraction was similar in all cases.

CYP2E1, P450R, and UGT2B7 were expressed most abundantly at 51.8, 31.6, and 55.0 pmol/mg protein of microsomal fraction, respectively (Table 1). The highest CV among samples was 76.1% for CYP2A6. The average of %CV of all samples was 43.4%. This is significantly greater than those of the individual donors ($P < 0.05$), which were 26.5%, 23.9%, and 23.0% for BD85, BD72, and BD87, respectively, suggesting that differences among the donors contribute substantially to the variances of expression levels of the target proteins in all samples. CYP2C9, 2C8, 2A6, 2C19, 2D6, 2B6, and P450R showed significant differences in protein expression levels among donors ($P < 0.05$). In addition, CYP3A5 and 3A7 were determined in all five samples from donor BD85, but were not detected or were detected in only 1 sample from the other donors. CYP2D6 was detected in only one sample from donor BD72, but was detected in all samples from BD85 and BD87.

Protein Expression Levels of Transporters in Plasma Membrane Fraction of PXB Mouse Liver. Protein expression levels of seven human ABC transporters, eight human solute carrier transporters, Na^+/K^+ ATPase, and γ-GTP were determined in plasma membrane fraction of PXB mouse liver, since these drug transporters function at the plasma membrane (Table 2). Na^+/K^+ ATPase and γ-GTP are membrane markers, and the CVs of their quantification values were 29.4% and 40.1%, respectively, among 11 samples. The quantified values were not significantly different among the three