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Microbes and Infection 16 (2014) 114–122

www.elsevier.com/locate/micinf

Original article

Retinoids and rexinoids inhibit hepatitis C virus independently of retinoid receptor signaling

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Received 8 August 2013; accepted 21 October 2013
Available online 28 October 2013

Abstract

Using a high-throughput screening system involving HCV JFH-1-Huh 7.5.1 cells, we determined that the ligands of class II nuclear receptors, retinoids and rexinoids inhibit HCV infection. Retinoids, ligands of retinoic acid receptor (RAR), and rexinoids, ligands of retinoid X receptor (RXR), reduced extracellular HCV RNA of HCV infected cells in a dose-dependent manner. The 50% effective concentrations were below 10 nM, and the 50% cytotoxic concentrations were over 10 μM. Both agonists and antagonists demonstrated inhibition, which indicates that the effect is not dependent on retinoic acid signaling. These chemicals reduced HCV RNA and NS5A protein levels in cells harboring the sub-genomic HCV replicon RNA, which suggests that the chemicals affect HCV RNA replication. These compounds were also effective against persistently infected cells, although the reduction in the intracellular HCV RNA was smaller than that of the extracellular HCV RNA, suggesting that viral post-replication step is also inhibited. In combination with interferon (IFN), retinoid exhibited a synergistic effect. Retinoids did not enhance expression of the IFN effector molecule PKR. These series of compounds warrant further investigation as new class of HCV drugs, for the clinical translation of our observation may lead to increased anti-HCV efficacy.

Keywords: Hepatitis C virus; HCV; Retinoid; Retinoic acid; Rexinoid

1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver diseases such as hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). HCC usually occurs after establishment of liver cirrhosis in HCV-infected individuals. Although the prevalence of HCV infection in HCC differs noticeably with geographical regions, two-thirds of hepatocellular carcinoma patients are chronically infected with HCV in Japan [1]. Because of limited efficacy and high cost of preexisting drugs, HCV infection has not yet been eradicated from the world especially Asia. The current standard therapy, a combination of interferon (IFN) and ribavirin, is not effective for all the patients, in addition to having serious side effects. Because of the urgent need for novel HCV therapies, many studies on HCV drugs have been conducted. In addition to *in vitro* screenings targeting specific HCV viral enzymes and screenings using HCV genome-harboring replicon cells, the HCV JFH-1-Huh 7.5 cell infection system has been recently developed [2] and is now used for screening. This system is applicable to easy screening assays [3,4] and is capable of identifying and analyzing inhibitors that have effects on any stages of HCV life cycle: viral attachment, entry, replication, and post-replication. This system targets not only viral components but also the host components involved in HCV infection.

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1286-4579/$ - see front matter
http://dx.doi.org/10.1016/j.micinf.2013.10.016

We screened chemical libraries using an easy high-throughput screening with the JFH-1-Huh 7.5 cell system [4], and discovered that several ligands of class II nuclear receptors inhibited HCV infection. These ligands had a notable effect on HCV infection; the 50% effective concentrations (EC_{50}) were below 10 nM. The nuclear receptors are classified into two classes: receptors for steroid hormones (class I) and receptors for non-steroid ligands (class II). The class II nuclear receptors include retinoic acid receptor (RAR), retinoid X receptor (RXR), peroxisome proliferator-activated receptors (PPARs), vitamin D receptor (VDR), thyroid hormone receptor (TR), and liver X receptor (LXR). The common biological characteristic of the class II receptors is that they work as a dimer with the RXR [5–7]. The RAR consists of three subtypes, α, β and γ, encoded by separate genes. All-*trans*-retinoic acid (ATRA) is a retinol (vitamin A) metabolite and considered as a natural ligand of RARs. ATRA and some synthetic analogs bind RARs and are referred to as retinoids [5,6]. ATRA is used as an effective anticancer drug for the treatment of acute promyelocytic leukemia (APL). Am80 is a synthetic retinoid with specific activation of RARα, which is also clinically used for APL as tamibarotene. RXR also consists of three subtypes, α, β, and γ. In addition, 9-*cis*-retinoic acid (9CRA) and some synthetic ligands of RXRs are called rexinoids [7]. RXR is unique in that it forms a homodimer, whereas all the other class II receptors exclusively form heterodimers with the RXR. 9CRA is also a metabolite of retinol and is believed to be a natural ligand of the RXR, but is capable of binding the RAR. Bexarotene is a synthetic selective agonist of RXR prescribed as Targretin and used for cutaneous T cell lymphomas in USA and some other countries.

There are some reports regarding the *in vitro* inhibitory effect on HCV of ATRA and other retinoids [8,9]. Furthermore, Böcher et al. reported a preclinical use of ATRA for the treatment of hepatitis C patients and demonstrated its therapeutic potential [10]. Nevertheless, the mechanism of the retinoid inhibitory effect on HCV has not been examined and remains unclear. Retinoids have been already approved for treatments of other diseases. That is, their pharmacological properties had been already investigated and safety is verified, suggesting benefit for HCV treatment. Therefore, in this study, we attempted to elucidate how retinoids and related chemicals inhibit HCV infection and obtain some clues allowing the understanding of the mechanism of action.

2. Materials and methods

2.1. Cells and virus

Huh 7.5.1 cells and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich Co. St. Louis, MO, USA) with 10% fetal bovine serum (FBS). HCV JFH-1 (genotype 2a) (HCVcc) was generated and stocked as described previously [3]. Subgenomic replicon cells, clone #4-1 and clone #5-15, which are derived from Huh 7 cells, harbor the JFH-1 genome (genotype 2a) [11] and the genotype 1b HCV genome [12], respectively. A persistently infected cell line was prepared as described below. Huh 7 cells were inoculated with HCVcc at a multiplicity of infection (MOI) of 1. The cells were passaged every 3–5 days with a cell density at more than 5×10^5 cells/10 cm-dish. HCVcc inoculated cells were monitored via intracellular and extracellular HCV core protein levels as determined by ELISA (HCV ELISA Test System, Ortho-Clinical Diagnostic K. K., Tokyo, Japan) and immunostaining and immunoblot using a specific antibody (anti-HCV core protein antibody, #40015B, Anogen, Mississauga, Canada). After HCVcc inoculation of Huh 7 cells, the intracellular HCV RNA and extracellular infectivity were detected for over ten passages. These cells were also cultured in DMEM with FBS.

2.2. Chemicals

All-*trans*-retinoic acid (ATRA) was purchased from Calbiochem-Merck KGaA (Darmstadt, Germany) and 9-*cis*-retinoic acid (9CRA) was from LKT (St. Paul, MN, USA). Am80, adapalene, TTNTB, and GW3965 were purchased from Tocris Bioscience (Bristol, UK). LE135, bexarotene, CD3254, UVI3003, SR11237, and TO901317 were purchased from Sigma–Aldrich Co. Ro41-5253, GW1929, and vitamin D3 were purchased from Enzo Life Science Inc. (Farmingdale, NY, USA). Clofibrate was purchased from Cayman Chemicals (Ann Arbor, MI, USA), and thyroxin was from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA). Human interferon (IFN) α was purchased from PeproTech Inc. (Princeton, NJ, USA).

2.3. Quantification of viral titer in the supernatant and cells

To detect the reduction of HCV RNA in the supernatant, we used an easy quantitative real-time RT-PCR, the tube-capture-RT-PCR described before [3]. Briefly, the test compounds were added to Huh 7.5.1 cells seeded in 96-well plates as was HCVcc (MOI = 0.01), and after 5 days, the culture supernatant was taken and subjected to quantitative real-time RT-PCR using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit™ (Invitrogen Co., Carlsbad, CA, USA) by the Agilent Technologies Mx3000P (Santa Clara, CA, USA) [3]. For measuring the copy number of HCV RNA, 5×10^4 cells were seeded in 24-well plate per well, infected, and cultured for the indicated time. RNA was extracted with the QIAamp viral RNA Mini Kit™ (Qiagen GmbH, Hilden, Germany) from the supernatant or with the RNeasy Mini Kit ™ (Qiagen) from cells for quantitative real-time RT-PCR. The primers for HCV RNA were 5′-GAGT GTCGTACAGCCTCCAG-3′ (nucleotides 97–116) and 5′-AGGCCTTTCGCAACCCA-3′ (nucleotides 280–264). The standard JFH-1 RNA protocol for measurement of copy number has been previously described [3]. As an internal control, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA was measured with the primers 5′-CCACCCATGGCAAATTCC-3′ and 5′-TGGGATTTCCATTGAT-3′. To evaluate the expression of protein kinase R (PKR) and 2′, 5′-oligoadenylate synthetase 1 (OAS1), we used 5′-TGGCCGCTAAACTTGCATATC-3′ and 5′-GCGAGTGTGCTGGTCACTAAAG-3′ as primers for PKR

and 5′-TGACGCTGACCTGGTTGTCTT-3′ and 5′-GAAC TCTCCCCGGCGATT-3′ as primers for OAS1.

Cell growth was monitored using an MTT assay, as described previously [13].

2.4. Western blotting

Western blotting was performed as previously described [3]. Briefly, cell lysates containing equal amounts of protein separated by SDS-PAGE were transferred onto PVDF membranes, and probed with antibodies against core (2H9), NS5 (Austral Biologicals, San Ramon, CA, USA), and GAPDH (Santa Cruz Biotech. Inc.).

2.5. Production and infection of pseudo-particles

The methods of production and infection of pseudo-particles have been previously described [14]. Briefly, HCV pseudo-particles (HCVpp) were generated with 3 plasmids: a Gag-Pol packaging construct, a transfer vector construct, and a HCV envelope glycoprotein-expressing construct (E1E2 of JFH-1), according to the method of Bartosch et al. [15] To produce pseudo-particles of vesicular stomatitis virus (VSV) (VSVpp), a plasmid coding VSV envelope, pCAG-VSV (kindly given by Dr. Y. Matsuura, Osaka University, Japan) was used instead of the plasmid expressing HCV envelope protein. The 3 plasmids were simultaneously transfected into 293T cells, and the supernatant was used as the pseudo-particle solution. For infection assay, the pseudo-particle solution with or without drug was inoculated onto Huh 7.5.1 cells. After 2-h incubation, the supernatants were changed to new complete medium, followed by 3-day incubation. Luciferase activities were assayed with the One-Glo™ luciferase assay system (Promega Co. Madison, WI, USA).

3. Results

3.1. Ligands of RAR and RXR inhibited HCV infection at nanomolar concentrations

Using an easy high-throughput screening for HCV inhibitors of the HCV JFH-1-Huh 7.5.1 cell infection system [4], we determined that some class II receptor agonists inhibit HCV infection. Therefore, we initiated further intensive study of the effect of various RAR and RXR agonists and antagonists. Huh 7.5.1 cells were infected with HCVcc at a MOI of 0.01 in the presence or absence of the chemicals. Five days after infection, the HCV RNA levels in the supernatant were measured by the tube-capture-RT-PCR. As shown in Fig. 1a, ATRA and all the synthetic retinoids we tested produced a marked reduction of HCV RNA in a dose-dependent manner, whereas the dose–response curves gradually lowered. The 50% effective concentrations (EC_{50}) were below 10 nM (Table 1). Notably, an antagonist of RARα, Ro41-5253 and an antagonist of RARβ, LE135 also reduced HCV RNA in a similar manner (Fig. 1a). We also examined the effects of various RXR ligands such as 9CRA, which is believed to be a natural agonist of the RXR and also an agonist of the RAR, synthetic RXR agonists (bexarotene, SR11237, and CD3254), and an RXR antagonist, UVI3003. All the RXR ligands we examined inhibited HCV RNA with a similar dose-dependent curve and the EC_{50} values were also below 10 nM (Fig. 1b and Table 1). The results demonstrated that although RXR ligands appeared to have a higher EC_{50}, both the agonists and antagonists of these receptors exhibited inhibitory activity on the production of extracellular HCV RNA. Given that the 50% cytotoxic concentrations (TC_{50}) of these compounds were over 10 μM, the specific indexes were over 1000 (Fig. 1a and b, and Table 1).

As the ligands of the two members of the class II nuclear receptors presented inhibitory effects, we attempted to examine some ligands of other class II receptors, such as PPAR, LXR, VR, and TR. These receptors form a heterodimer with RXR but do not form the respective homodimer. A PPARα agonist clofibrate did not exhibit any inhibition, and although a PPARγ agonist GW1929 showed some inhibition, there was no concentration dependence (Fig. 1c). LXR agonists GW3965 and TO901317 displayed inhibition in a dose-dependent manner, although the EC_{50} of these drugs were higher, in that they were between 10 nM and 1 μM (Fig. 1c). Thyroxin and vitamin D3 did not inhibit HCV infection at these concentrations (Fig. 1c). These results demonstrated that not all the class II receptor ligands have an inhibitory effect on HCV infection. The RAR and RXR ligands exhibited a robust inhibitory effect, whereas LXR agonists displayed a weak dose-dependent inhibition.

Next, we examined the intracellular and extracellular HCV RNA amount in the presence of Am80 for 5 days. The Huh 7.5.1 cells were infected with HCVcc at a MOI of 0.01 in the presence of 10 μM Am80 and incubated, and the RNA was extracted from the supernatant and the cells every day for 5 days. The viral RNA copy number was increased, but inhibition was observed in the presence of 10 μM Am80 (Fig. 1d). Am80 gradually attenuated the increase of HCV RNA both in the supernatant and in the cells with the passage of days. On day 2 post-infection, the relative amount of extracellular HCV RNA to the control was 54% (Fig. 1e). However, the relative amount of extracellular HCV RNA fell to 7% of the control level on day 5 post-infection, although the intracellular HCV RNA level decreased to 23% of the control level (Fig. 1e). It took 5 days until full repression of HCV infection was observed, and the viral release from the cells appeared to be inhibited during this period.

We also examined the production of HCV core protein in cells using western blotting. As expected, the tested chemicals inhibited the core protein expression in the cells (Fig. 1f).

3.2. Retinoids and rexinoids inhibited HCV RNA replication but not entry

To know the mechanism of inhibitory action we first examined the effect on viral entry of these compounds using pseudo-particles. HCVpp or VSVpp solution was mixed with the drug and inoculated into Huh 7.5.1 cells for 2 h. Then the cells were washed and supplied fresh medium without drug, and we performed a luciferase assay after a 3-day culture. The

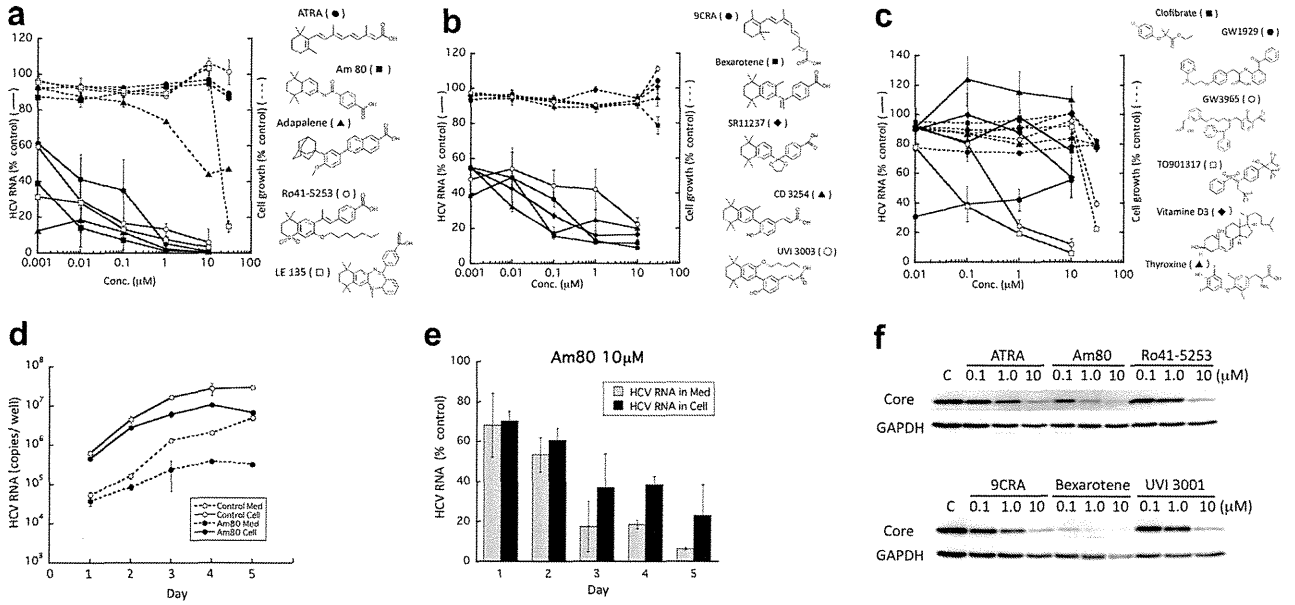

Fig. 1. Effects of agonists and antagonists of RAR and RXR on JFH-1 HCV infection. Huh 7.5.1 cells infected with HCVcc (MOI = 0.01) in the presence of drugs were incubated for 5 days. HCV RNA in the supernatant was measured by tube-capture RT-PCR previously described [3]. Parallel cultures of cells without virus were analyzed using an MTT assay to detect the inhibition of cell growth due to drug exposure. The percentages to control HCV RNA and control cell growth are indicated by solid lines and dotted lines, respectively. (a) Effects of RAR agonists and antagonists. ATRA (all-*trans*-retinoic acid, closed circles), Am80 (closed rectangles) and adapalene (closed triangles) are agonists of RAR. ATRA has no subtype specificity. Am80 and adapalene binds specific to RARα and RARβ, respectively. Ro41-5253 (open circles) and LE135 (open rectangles) are antagonists of RARβ and RARα, respectively. (b) Effects of RXR agonists and antagonists. 9CRA (9-*cis*-retinoic acid, closed circles) is an agonist of both RAR and RXR. Bexarotene (closed rectangles), SR11237 (closed rhombus), and CD3254 (closed triangles) are RXR agonists, whereas UVI3003 (open circles) is an RXR antagonist. (c) Effects of other class II nuclear receptor ligands. Clofibrate (closed rectangles) and GW1929 (closed circles) are PPARα and PPARγ agonists, respectively. GW3965 (open circles) and TO901317 (open rectangles) are LXR agonists. Vitamin D3 and thyroxin are indicated by closed rhombus and closed triangles, respectively. The values are the averages of triplicates, and the error bars represent the standard deviation. (d) Time-dependent expression profile of the HCV RNA in the supernatant and in the cells. Huh 7.5.1 cells were infected with HCVcc (MOI = 0.01) in the presence or absence of 10 μM of Am80 and incubated for 5 days. HCV RNA was extracted from the supernatant and from cells every day and subjected to quantitative real-time RT-PCR. The values indicate the copy numbers/well of 24-well plate. (e) Time-dependent expression profile represented by percentages of those in untreated cells based on (d). The results are represented by percentages of the levels of untreated cells. The values are averages of triplicates and the error bars represent the standard deviation. (f) Huh 7.5.1 cells were infected (MOI = 0.01) in the presence of retinoids and incubated for 5 days. The cell lysates were blotted with anti-core antibody and anti-GAPDH antibody.

luciferase activity of the cells infected with HCVpp was elevated and was not inhibited by retinoids or rexinoids (Fig. 2a). The luciferase activity of VSVpp was also not reduced at these concentrations (Fig. 2a). These results indicate that these drugs did not affect the viral entry.

Next, to examine whether these drugs inhibit the viral replication step, we conducted experiments using subgenomic replicon cells. The subgenomic replicon is a special cell system in which HCV RNA replicates autonomously and expresses viral proteins without viral entry or release. We treated subgenomic replicon cells derived from Huh 7 cells that is harboring the JFH-1 genome (#4-1, genotype 2a) with retinoids and rexinoids for 6 days at the indicated concentrations and measured the amount of cellular replicon RNA. Although the effective concentration was considerably high (EC_{50} of Am80 was 6 μM), the HCV RNA was reduced by the treatment with the compounds in a dose-dependent manner compared with GAPDH mRNA expression (Fig. 2b). Furthermore, we examined the effects of the drugs on HCV NS5A protein expression in the replicon cells by western blotting. These drugs, except ATRA, reduced the NS5A expression of the cells at a concentration of 30 μM but not the GAPDH protein (Fig. 2c, upper figure). We also examined the expression of NS5A protein in another subgenomic replicon cell line (#5-15) that harbors a genotype 1b HCV replicon. Treatment for 5 days with 30 μM of the retinoid and rexinoid compounds, except for Am80, resulted in a decrease in NS5A protein expression without affecting the amount of cellular GAPDH. However, in addition to Am80, 10 μM ATRA and bexarotene appeared to increase NS5A expression (Fig. 2c, lower figure). Although the protein level is influenced by metabolism or subcellular modification,

Table 1
EC_{50} and TC_{50} of retinoids and rexinoids.

	EC_{50} (nM)[a]	TC_{50} (μM)	TC_{50}/EC_{50}
ATRA	4.45	30<	6000<
Am80	0.00647	30<	4,000,000<
Adapalene	0.001>	20	20,000,000<
Ro41-5253	0.943	30<	30,000<
LE135	0.00477	20	4,260,000
9CRA	4.24	30<	7000<
Bexarotene	0.190	30<	100,000<
SR11237	1.45	30<	20,000<
CD3254	0.260	30<	100,000<
UVI3003	5.68	30<	5000<

[a] EC_{50} were calculated by equations of logarithmic fitting curves based on the data in Fig. 1a and b.

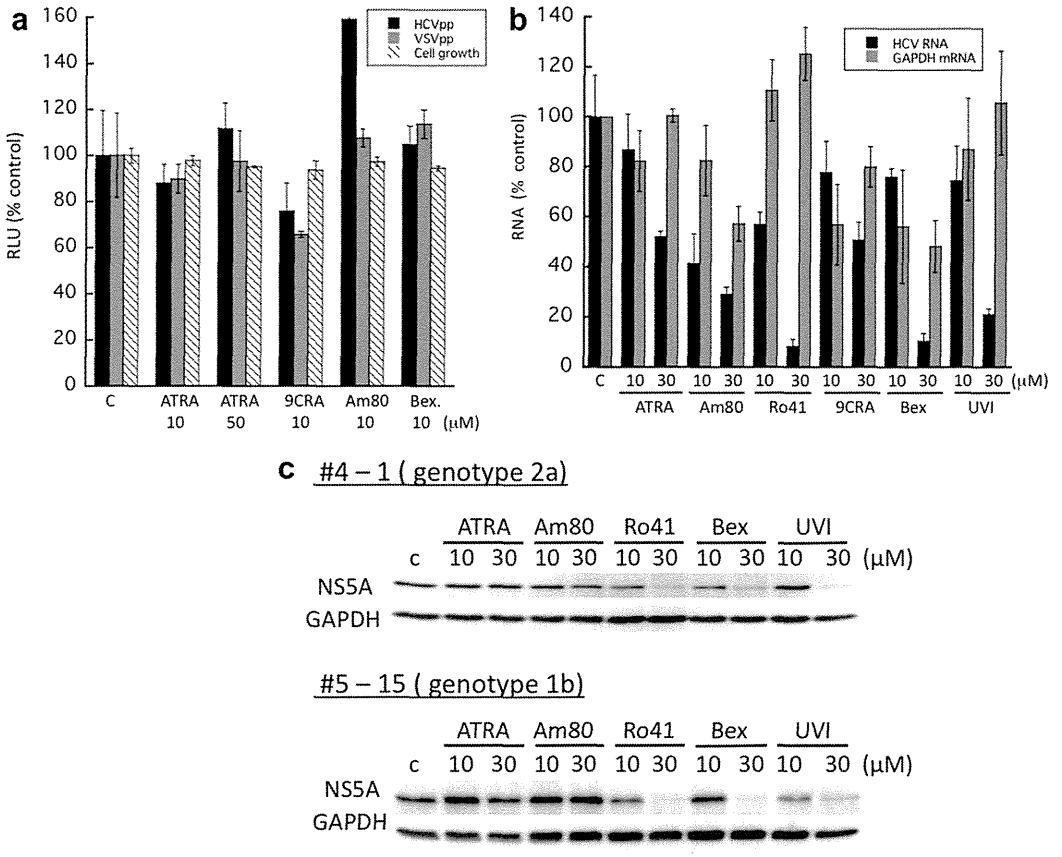

Fig. 2. Effects of retinoids and rexinoids on infection of HCV pseudo-particles and subgenomic replicon cells. (a) Huh 7.5.1 cells received HCVpp mixed with drug, and the medium was replaced after 2 h, followed by culturing for 3 days. Parallel infection of VSVpp with drugs was performed to compare the effects. Another parallel culture without pseudo-particles was evaluated by MTT assay for detecting effects on cell growth. The values are the averages of triplicates, and the error bars represent the standard deviation. (b) Subgenomic replicon cell harboring genotype 2a (JFH-1), #4-1 was treated with retinoids for 6 days. Total RNA was extracted from the cells and applied to quantitative real-time RT-PCR using primers for HCV or GAPGH. (c) Subgenomic replicon cell #4-1 (JFH-1, genotype 2a) or #5-15 (genotype 1b) were treated with the drugs for 7 days or 5 days, respectively. The cell lysates were applied to western blotting with anti-NS5A antibody or anti-GAPDH antibody. Ro41, Ro41-5253; Bex, bexarotene; UVI, UVI3003.

NS5A protein expression depends on the viral replication in the replicon cells. Therefore, the reduction in NS5A protein suggested that these compounds inhibited molecule(s) involved in HCV replication. The cell growth of the replicon cells was not suppressed at 30 μM or less concentrations of all the compounds (data not shown), indicating that the reduction in the viral protein level does not result from cell toxicity. In conclusion, although the effective concentrations were markedly higher compared with those in the JFH-1-Huh 7.5.1 cell system, the tested compounds inhibited HCV RNA replication. In addition, the compounds were effective against not only HCV genotype 2a but also genotype 1b.

3.3. Effect of retinoids on persistently infected cells

To investigate the possibility that retinoids are effective on chronic hepatitis C, we used HCV-persistently infected cells. We had established the cell line as described in the 'Materials and methods'. The cell is derived from Huh 7 cells, and constitutively infected with HCV JFH-1, and the control intracellular HCV RNA and extracellular HCV RNA were 4.4×10^6 copies/well and 3.5×10^5 copies/well of 24-well plate, respectively, in the present experiment. Although the effective concentrations were higher (3–30 μM) than those in the JFH-1-Huh 7.5.1 infection experiment, the amount of HCV RNA was reduced in both the supernatant and the cell by retinoid treatment (Fig. 3). These results suggest that retinoids may be useful in the treatment of chronically infected hepatitis C. Smaller reduction in the intracellular HCV RNA compared with extracellular HCV RNA, which corresponds to the results of the JFH-1-Huh 7.5.1 cell system in Fig. 1e, suggests that retinoids also inhibited the viral post-replication step. In contrast, IFNα (10 unit/ml), which is believed to inhibit the viral replication, exhibited reduction in extracellular HCV RNA equivalent to those in intracellular HCV RNA (Fig. 3).

3.4. Effect of drug combinations on HCV infection

When considering the application to clinical use of retinoids and rexinoids, we attempted to access the effect of combination treatment on the JFH-1-Huh 7.5.1 cell system. First, we treated Huh 7.5.1 cells with the indicated concentrations of Am80 and bexarotene together, followed by viral inoculation (MOI = 0.01) and incubation for 5 days. Then, we

lysed the cells to evaluate the intracellular core protein expression using western blotting. As shown in Fig. 4a, Am80 and bexarotene singly inhibited the expression of HCV core protein; however, a combined treatment produced a more marked reduction, that is, an additive or synergistic effect.

Although the antagonists were solely effective against HCV infection, we next attempted to examine the effect of the combination of RAR agonists and antagonists. An RAR agonist, TTNTB, in combination with an antagonist, Ro41-5253, also displayed an additive or synergistic inhibition of HCV core protein expression (Fig. 4b). This result supports that the inhibitory effect on HCV infection by retinoids is independent of the RARE (retinoic acid response element) signal mediated by RAR-RXR. Such combination treatments might be useful for HCV therapy because the antagonist would counteract other unfavorable RARE-dependent effects.

Furthermore, as IFNα is used as a standard medicine for the treatment of hepatitis C, it would be useful if retinoids exert an additive or synergistic effect on IFNα treatment. When we performed western blotting with IFNα and Am80 under similar conditions, the HCV core protein in the IFNα (1.0 unit/ml)-treated cells also displayed a marked reduction in the existence of 0.1 μM Am80 (Fig. 4c). To determine whether the combined administration of retinoid and IFNα is additive or synergistic inhibition, we examined the effect on HCV RNA in the presence of the two drugs. We added the various concentrations of IFNα in combination with Am80 into Huh 7.5.1 cells, followed by viral inoculation (MOI = 0.01). After 5 days, HCV RNA was extracted from the supernatant, and the amount was measured using real-time RT-PCR. The result revealed that IFNα and Am80 singly reduced HCV RNA to 90% of the levels observed in the control at a concentration of 14.5 unit/ml and 0.192 μM, respectively (Fig. 4d). Combinations of 5.0 unit/ml IFNα with 0.0001 μM Am80, and 2.5 unit/ml IFNα with 0.001 μM Am80 demonstrated 90% inhibition of HCV RNA. The isobologram analysis indicated that the combined administration of IFNα and Am80 exhibited a synergistic effect (Fig. 4d). These results demonstrate the possibility that a combination treatment of retinoids and IFNα is useful for HCV treatment.

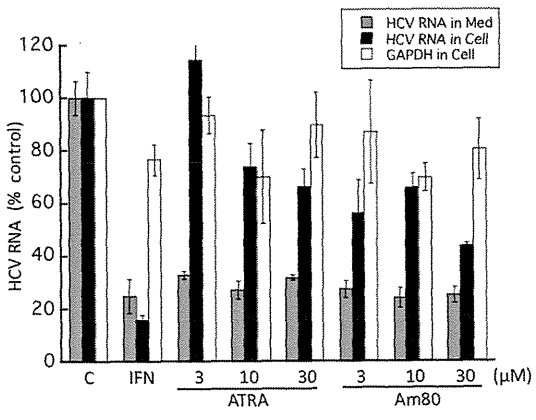

Fig. 3. Effects of retinoids on persistently HCV-infected cells. Persistently HCV-infected cells were treated with indicated concentrations of retinoids or IFN (10 unit/ml) for 5 days. HCV RNA was extracted from supernatant or cells. Quantitative real-time RT-PCR was performed with primers for HCV or GAPDH. The values are the averages of triplicates and the error bars represent the standard deviation.

3.5. Retinoids did not activate the IFN signaling effector molecule PKR

Retinoic acid reportedly activates the expression of some molecules, which play central roles in IFN signaling [16,17]. We attempted to investigate if retinoids inhibit HCV replication through IFN signaling. As there are multiple pathways involved in IFN signaling, we examined the expression of the IFN signaling downstream antiviral effector molecules, OAS1 and PKR. These molecules are present ubiquitously at constitutive levels but are increased and activated by IFN and play critical roles in the degradation of HCV RNA [18,19]. We treated Huh 7.5.1 cells with 10 unit/ml IFNα for 48 h and the cellular RNA was extracted. The level of PKR mRNA in the IFNα-treated cells was approximately 4-fold higher than that of the untreated cells, whereas that of OAS1 was 1.7-fold higher (Fig. 5). These results suggest the possibility that PKR considerably contributes to the antiviral effect of IFNα in the examined cells. We treated the cell with 10 μM of Am80, bexarotene, or Ro41-5253 for 48 h, extracted RNA from the cell, and estimated the RNA expression levels of OAS1, PKR and GAPDH using quantitative real-time RT-PCR. The relative RNA levels compared with the control were calibrated by a standard curve of RNA extracted from IFNα (10 unit/ml)-treated cells. The three chemicals at the concentration of 10 μM, produced no enhancement of PKR expression and a slight enhancement of OAS1 expression (1.2–1.3-fold) (Fig. 5). GAPDH expression of the cells was slightly enhanced by the three compounds and IFNα. Although there were some increases in OAS1 expression, it is unlikely that these compounds inhibited through enhancement of OAS1. These results, when taken together, suggest that retinoids and rexinoids presumably inhibit HCV infection through some other modes of action than the activation of IFN signaling.

4. Discussion

This study revealed that RAR and RXR ligands inhibited HCV infection at significantly low concentrations (EC_{50} <10 nM) in the JFH-1-Huh 7.5.1 cell-culture system (Fig. 1 and Table 1). Until now, there have been a few reports concerning the *in vitro* or *in vivo* effect of retinoids on HCV infection. Regarding *in vitro* inhibition, the RAR agonists, TTNPB and 13-*cis*-retinoic acid, were determined to have an inhibitory effect in screening studies [8,9], whereas a slight activation by TTNPB in a HCV replicon system was also reported [20]. On the other hand, there had been some reports of retinoids being used for preclinical application against hepatitis C. Böcher et al. reportedly treated HCV patients who were resistant to IFNα with ATRA and obtained good results, especially in combination with IFNα [10]. Kohge et al. reported a pilot study involving retinol in combination with

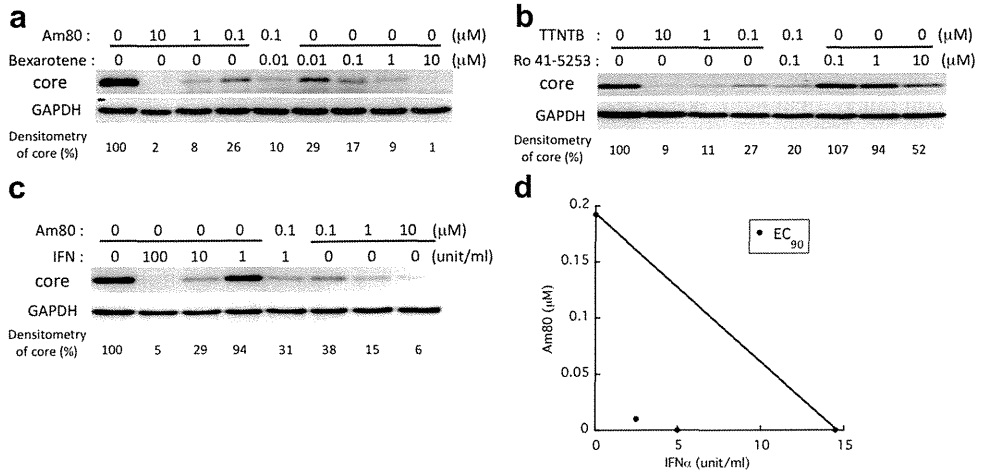

Fig. 4. Effects of combined application on HCV infection. with Huh 7.5.1 cells were infected with HCVcc at a MOI of 0.01 in the presence of (a) Am80 and bexarotene, (b) TTNTB and Ro41-5253, and (c), (d) Am80 and IFNα. After 5 days of incubation, the cells were lysed and subjected to western blotting with anti-core antibody and anti-GAPDH antibody (a, b, and c), or RNA was extracted from the supernatant, applied to real-time RT-PCR, and the EC_{90} were plotted in an isobologram (d). The relative amounts of core protein to the untreated cells (control) were determined using ImageJ and described under the figures (a–c).

IFNα-ribavirin to chronic hepatitis C, and they obtained some positive results from the combined treatment [21].

Nevertheless, the inhibitory mechanism of HCV replication by retinoid has been scarcely studied. In this study, we performed some experiments to clarify the mechanism or target molecule(s) of retinoids and rexinoids. Although we were not able to provide an explicit mechanism of action, this study identified some suggestions that implicate the mechanism. First, these compounds affect some of the molecules involved in HCV viral replication and post-replication steps (Figs. 1e, 2, and 3). Second, the mechanism is not related to the RARE-dependent signaling mediated by RAR/RXR because the antagonists also demonstrated inhibition of HCV infection and further additive/synergistic effects (Fig. 1a, b, and 4b). Third, these compounds appeared to inhibit HCV via a mechanism other than IFN signaling because they could not enhance the expression of the IFN effector molecule PKR (Fig. 5).

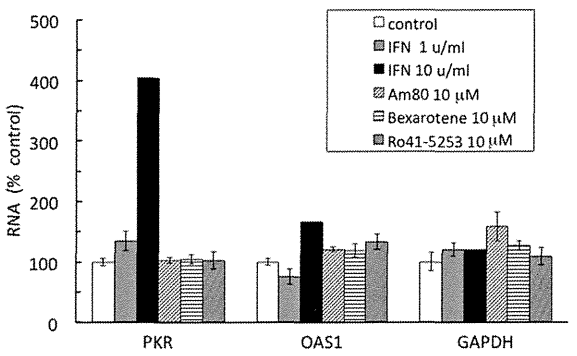

Fig. 5. Effects of retinoids and rexinoids on the expression of PKR, OAS1, and GAPDH. Huh 7.5.1 cells were treated with IFN, Am80, bexarotene, or Ro41-5253 for 48 h. RNA was extracted from the cells and subjected to real-time RT-PCR. The relative amount of PKR, OAS1, and GAPDH mRNA are represented by percentages relative to the values observed with untreated cells. The values are the averages of triplicates and the error bars represent the standard deviation.

As shown in Fig. 1, dose-dependent inhibition was observed in the cells treated with retinoid, rexinoid, and LXR ligands but not in the cells treated with PPAR ligands, vitamin D3, or thyroxin. Although RXR is common partner of these class II receptors, not all of the class II ligands inhibited HCV infection, suggesting that the RXR is not the main target of the HCV inhibitory effect. The finding that vitamin D3 or thyroxin did not inhibit HCV infection might be explained by the absence of the expression of VDR or TR in the tested liver cells. However, as PPARα is generally expressed in liver cells, it was deduced that RXR is not the target molecule of retinoids in regards to HCV inhibition. Furthermore, the fact that antagonists have inhibitory activity as well as agonists suggested that the effect is not mediated via RAR-RXR signaling.

After the report of Böcher et al., Kast believed that the inhibitory effect of retinoids would be explained by the enhancement of IFN signal via RIG-I (retinoic acid inducible gene I) [22]. RIG-I is an intracellular sensor molecule that recognizes HCV dsRNA to activate IFN signaling [23]. However, there is a retinoid-binding site in the promoter domain of RIG-I gene, and RIG-I expression requires RARE-dependent signaling [24]. In addition, the Huh 7.5.1 cells reportedly are defective for RIG-I signaling by a point mutation in this gene [25]. Retinoids also modulate expression of STAT1 (signal transducer and activator of transcription 1) and IRF-1 (interferon regulatory factor-1) [26–28], which also play critical roles in the IFN system during HCV infection [29,30]. The STAT1 gene has a RARE motif in the promoter, and the enhancement is dependent on RAR-RXR signaling [26], whereas IRF-1 expression was not mediated through the RARE motif [31].

As the IFN system is highly complicated, we examined the possibility that the IFN system is involved in the retinoid activity by examining the expression of the effector molecules PKR and OAS1. Since PKR expression was enhanced 4-fold by 10 unit/ml IFNα, the main effector molecule appeared to be PKR in the cells. However, 10 μM of retinoids, with

antiviral activity corresponding to 10 unit/ml IFNα, produced no enhancement of PKR (Fig. 5). These findings suggest that these compounds inhibited HCV infection mainly via a mechanism other than the IFN signaling.

This study suggested that there are two target steps of viral lifecycle affected by retinoids and rexinoids: the HCV replication step and the post-replication step. Given that the compounds reduced the HCV RNA of the subgenomic replicon cells, the HCV viral replication step appears to be inhibited (Fig. 2). Based on the observation that the intracellular HCV RNA was less reduced compared with extracellular HCV RNA (Figs. 1e and 3), the post-replication step also appears to be inhibited. On the other hand, higher concentrations of ligands were required to affect the subgenomic replicon cells (Fig. 2b and c). This suggests that the post-replication step might be the major target rather than the replication step. If so, some molecules critical for viral maturation or release may be disturbed. Some common structures of these compounds might interact with such a target molecule. However, a higher concentration of retinoids is also required for the effect on the persistently infected cells (Fig. 3). Given that both the subgenomic replicon cells and the persistently infected cells are derived from Huh 7 cells, the lower sensitivity of these cells might be the result of some property of Huh 7 cells (e.g., the abundance of or accessibility to the target molecule involved in HCV replication).

Although the mechanism of the inhibitory effect is not clear, the results in this study revealed the usefulness of the combination of retinoids. The combination treatment of agonists and antagonists is useful because the antagonists can counteract the unfavorable effects other than the anti-HCV activity of retinoids. Treatment with retinoids in combination with IFNα also has further potential. Given that Am80 and bexarotene are already used clinically to treat other diseases, and many agonists and antagonists of nuclear receptors II are being developed [5,7,32], further studies of retinoids and rexinoids for practical application against HCV are warranted.

Acknowledgments

We thank Dr. Koichi Watashi for helpful advice. We also thank Dr. Yoshiharu Matsuura (Osaka University, Japan) for generously providing plasmids. This study was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan.

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抗体医薬品の分子設計

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1. はじめに

マウスモノクローナル抗体作製技術の開発を発端に，キメラ抗体，ヒト化抗体，ヒト抗体と進化した抗体医薬品は，IgGサブクラス置換，アミノ酸置換，糖鎖改変，薬物修飾，低分子化，PEG化等の分子設計技術の応用により，多様化している．抗体医薬品の分子設計では，目的とする適応疾患，剤形，投与経路等を念頭に，有効性・安全性を得るために必要な薬理作用，薬物動態，ならびに，製剤化を考え，構造の至適化が進められるが，その他に，有効性低下や有害反応発生につながる可能性のある免疫原性，さらには，製造工程についても考慮する必要がある．本稿では，IgGの構造と機能に基づき，抗体医薬品の薬理作用及び薬物動態に関して概説した上で，抗体医薬品の分子設計においてポイントと考えられる事項を述べ，生物薬剤学の観点で重要となる薬物動態の至適化を目的とした分子設計の例を紹介する．

2. 抗体医薬品とは

抗体医薬品は，免疫グロブリンを医薬品としたものである．古くからヒト血漿より精製した免疫グロブリン製剤が用いられていたが，近年，開発が盛んな抗体医薬品は，ハイブリドーマ法やファージディスプレイ法等を利用して作製されたモノクローナル抗体をリード抗体とし，必要に応じて，様々な分子設計に基づく改変を施したものである．

2.1 IgGの構造と機能

図1にヒトIgG1の構造と機能を示す．IgG1は，2本のH鎖及び2本のL鎖からなる分子量約150,000の糖タンパク質で，CH2ドメインのAsn297にN-結合型糖鎖付加部位が存在する[1]．可変部の配列が各抗体により異なり，可変部に含まれる相補性決定部（CDR）が抗原結合に関わる．定常部は，遺伝子多型による数個のアミノ酸残基の違いを除き，IgGサブクラスが同じ抗体に共通する配列である．可変部と定常部の間はヒンジ部と呼ばれ，H鎖間のジスルフィド結合が位置する（図1A）．

ヒンジ部の一部，CH2，及びCH3ドメインからなるFcドメインは，Fcγ受容体や補体との結合能を持ち，Fcγ受容体の活性化による抗体依存性細胞傷害（ADCC）活性，及び，補体の活性化による補体依存性細胞傷害（CDC）活性に関与している（図1B-i）．

また，Fcドメインは，IgGの輸送担体である新生児型Fc受容体FcRnとの結合能を持ち，FcRnによるリサイクリングあるいはトランスサイトーシスに関与する[2]（図1B-ii）．非特異的飲作用であるピノサイトーシス等により細胞に取り込まれたIgGは，エンドソーム内でFcRnに結合し，細胞外にリサイクルされる．この機構により，IgGがリソソームへの輸送と分解を免れるため，ヒト生体内IgGの血中半減期は約20日と極めて長い．IgGは，FcRnにより

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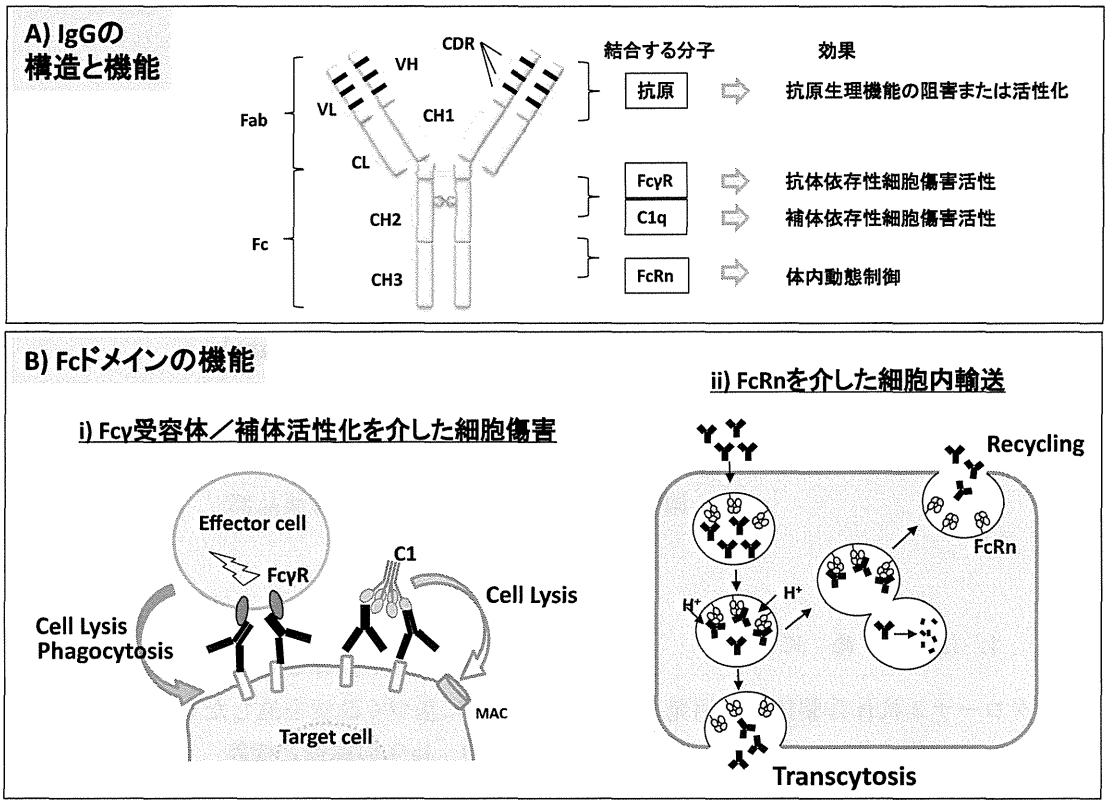


図 1　IgG の構造と機能

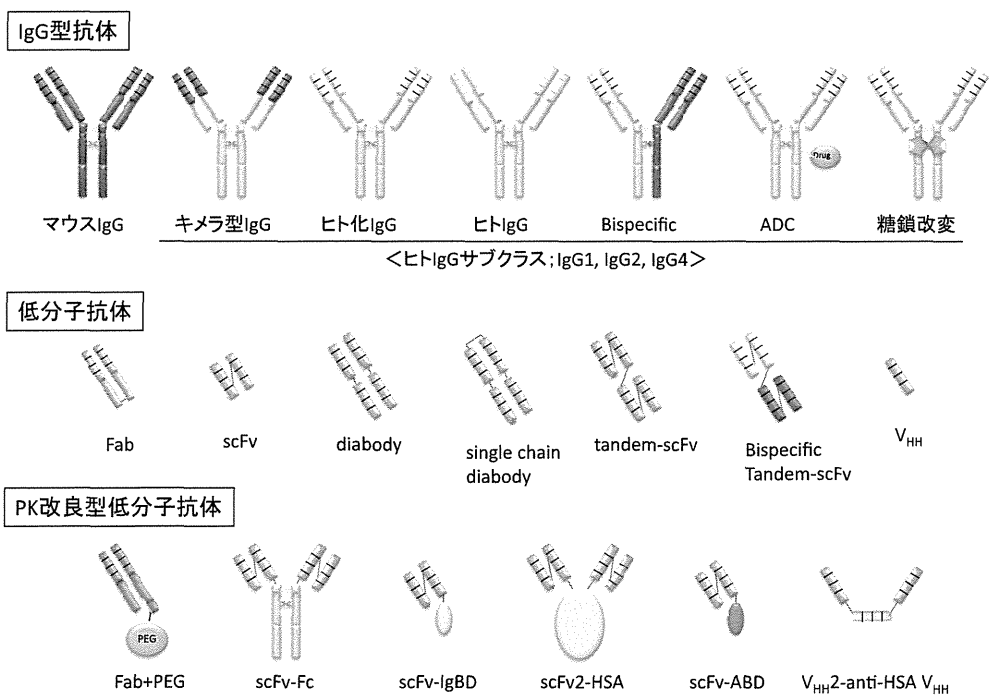


図 2　抗体医薬品の骨格構造の例

トランスサイトーシスされることも知られており，胎盤では，IgG が FcRn を介して母親から胎児に輸送される．

2.2　抗体医薬品の構造

図 2 に，IgG 型抗体，低分子抗体，PK 改良型低分子抗体に分類して，抗体医薬品の骨格を図示した．IgG 型抗体には，典型的な IgG 型抗体としてマウス抗体，キメラ抗体，ヒト化抗体，ヒト抗体があり，その他に，二重特異性抗体，抗体薬物複合体，糖鎖改変抗体，アミノ酸配列改変抗体等がある．キメラ

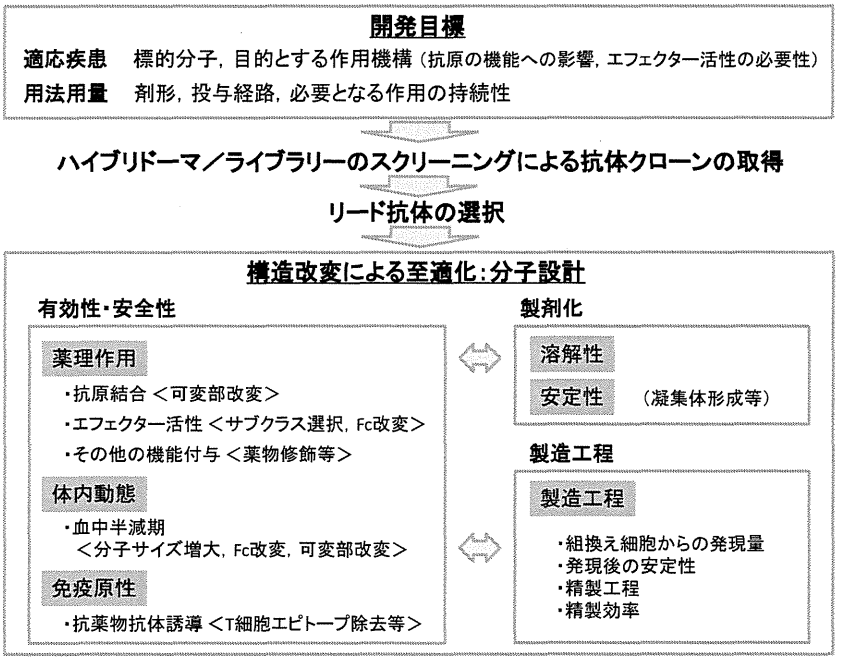


図3　開発目標に応じた抗体医薬品の分子設計において考慮すべき主な事項

抗体は，マウス抗体の定常部をヒト抗体に置換したもの，ヒト化抗体は，マウス抗体のCDR以外を全てヒト抗体に置換したものである．キメラ抗体やヒト化抗体は，マウスIgG配列をヒトIgG配列に置換することにより，免疫原性の低減とヒトFcRn結合能の付与を実現したもので，抗体医薬品の実用化に大きく貢献した分子設計である．

低分子抗体には，可変部と定常部CL及びCH1ドメインからなるFabの他，可変部のみからなるscFv，2つのscFvが会合したdiabody，2つのscFvをリンカーでつないだtandem-scFv，1本鎖で抗原結合能を持つラマ由来抗体可変部V_{HH}などがある．これら低分子抗体においても，キメラ化やヒト化等，免疫原性を低減する改変が行われている．また，低分子抗体では，PEG化やFcRn結合性の付与等，血中半減期延長に寄与する修飾が行われることがあり，図2では，これらをPK改良型低分子抗体として示している．現在のところ，日米欧で承認されている抗体医薬品の中で，低分子抗体は，Fabが2品目，PEG化Fab′が1品目であり，IgG型抗体と比較すると少ない．抗体医薬品をはじめ，バイオ医薬品の承認品目については，国立医薬品食品衛生研究所生物薬品部HPにて情報提供している（http://www.nihs.go.jp/dbcb/mabs.html）．

3. 抗体医薬品の分子設計において考慮すべきこと

開発目標に応じた抗体医薬品の分子設計において重要と考えられる主な事項を図3にまとめた．抗体医薬品の開発では，まず，目的とする適応疾患に応じて抗原が選択され，ハイブリドーマやファージディスプレイライブラリー等のスクリーニングにより，目的とする抗原への結合能を持つ抗体クローンが取得される[1]．得られた抗体クローンの中から，抗原との結合親和性や特異性，及び，抗原の生理機能への影響を評価して，開発候補となるリード抗体が選択される[1]．

選択されたリード抗体の至適化においては，(1) 有効性・安全性に関連する薬理作用，薬物動態，免疫原性，(2) 製剤化に関連する溶解性，安定性，さらに，(3) 製造工程を考慮して，構造の至適化が行われる．

3.1　有効性・安全性

3.1.1　薬理作用

抗体医薬品の薬理作用は，抗原との結合，及び，エフェクター活性に寄与するFcγ受容体や補体との結合に依存するため，これらの結合能を至適化するための改変が行われる．また，目的とする薬理作用に応じ，二重特異性抗体への改変や，化学薬品による修飾等が行われることもある．

(1) 抗原結合の至適化

抗原結合には，可変部の構造が関与する．リード抗体の抗原結合親和性が不十分な場合や，ヒト化に伴い親和性が低下した場合，あるいは，特異性の向上が必要となる場合，CDR及びその周辺のフレームワーク部のアミノ酸置換が行われる[3]．

1つの抗体が2種類の抗原に結合することで薬理作用の発揮が期待できる場合，1つの抗体に2種類の可変部を持たせ，二重特異性抗体とすることがある[4]．二重特異性抗体の例として，腫瘍細胞表面抗原とT細胞表面抗原に結合する抗体があり，腫瘍細胞近傍でT細胞を活性化することで，抗腫瘍効果を示す．

(2) エフェクター活性の至適化

ADCC活性やCDC活性等のエフェクター活性には，Fcドメインのアミノ酸配列及び糖鎖構造が関与している．通例，細胞傷害活性を期待する抗体医薬品ではエフェクター活性を増強，中和活性のみを期待する抗体医薬品ではエフェクター活性を低減する方向で改変が行われる．

エフェクター活性を考慮した至適化において，まず，IgGサブクラスの選択が行われ，さらに，必要に応じて，Fcγ受容体や補体結合に関与するアミノ酸残基の改変が行われる．ヒトIgGには，IgG1～4のサブクラスがあり，これまでに承認されている抗体医薬品の多くでは，IgG1サブクラスが用いられているが，IgG2, IgG4，あるいは，IgG2と4のキメラ定常領域が用いられている例がある．IgG4はエフェクター活性が弱く，特に補体活性化能が低い点が特徴で，中和のみを目的とする抗体に選択される．IgG3はエフェクター活性が強いという特徴を持つが，ヒンジ領域が長く分子間ジスルフィド結合の数が多いことや，遺伝子多型が多いこと等が懸念され，これまでのところ，抗体医薬品に使われている例はない．

糖鎖構造改変の例として，Asn297に結合するN結合型糖鎖において，フコシル化された糖鎖の含量を低減することでFcγRIIIへの結合親和性を上げ，ADCC活性を増強する技術が日本で開発されている．抗CCR4抗体モガムリズマブがこの例である(表1)．

(3) 化学薬品による修飾

抗腫瘍効果を期待する抗体医薬品では，抗体と強力な細胞傷害作用を持つ薬物を共有結合させた抗体薬物複合体（ADC）として開発されることがある．細胞表面抗原に結合したADCは，抗原の細胞内移行に伴いエンドソームに移行し，酸加水分解，酵素消化等により，薬物が放出される．薬物の放出性はリンカーの構造に依存するため，ADCの分子設計においてはリンカーの設計が重要である．

3.1.2 薬物動態

化学薬品では，薬物動態の制御における製剤設計の重要性が高いが，抗体医薬品では，有効成分の構造が薬物動態に関わるため，その分子設計において，薬物動態を考慮することになる．抗体医薬品は，それ自身が標的指向性を持っているため，薬物動態に関しては，血中滞留性や組織移行性が課題となる．本章第4節（抗体医薬品の体内動態制御のための分子設計）で述べるように，IgG抗体では，Fcドメインの改変によるFcRn結合親和性向上や，可変部の改変による遊離型抗体のリサイクリング等を目的とした分子設計が行われている．低分子抗体では，主に，血中半減期延長のための分子設計が行われる．

3.1.3 免疫原性

免疫原性は，*in vivo*で免疫応答を生じさせる性質であり，抗体医薬品を含むバイオ医薬品の有効性・安全性確保に関する懸念事項の一つとなっている．投与された医薬品が免疫原性を示し，抗薬物抗体が産生されると，薬物の血中半減期への影響や，有効性の低下，免疫応答による有害作用発生につながる可能性がある．ヒトに対する免疫原性の程度は，臨床試験を実施しなければ分からないが，臨床試験段階で免疫原性の問題が生じると，開発の続行が危ぶまれるため，分子設計の段階で，免疫原性に寄与する構造についても考慮する．

免疫原性の回避について，今のところ定型化された手法はないが，分子設計に寄与する情報を得る方法として，抗原提示に関わるMHCクラスII分子に結合するペプチド配列（T細胞エピトープ）を推定することや，リード抗体選択に際し，ヒトT細胞の活性化を指標とした*in vitro*アッセイを利用すること等が考えられる．

3.2 製 剤 化

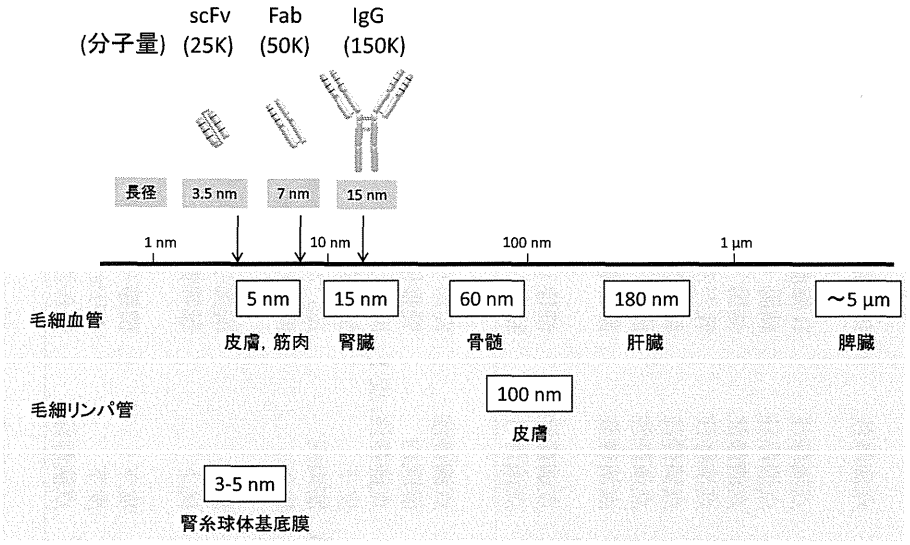
3.2.1 溶解性

これまでに承認されている抗体医薬品は，全て注射剤であり，投与経路は，抗腫瘍薬では点滴静注，

表1　日本で承認された抗体医薬品

	構造	標的分子	一般名	販売名	剤形	投与経路	主な適応疾患
抗腫瘍薬							
マウス	IgG1κ (MX-DTPA：^{90}Y 標識)	CD20	イブリツモマブ チウキセタン	ゼヴァリン イットリウム	溶液	点滴静注	B 細胞性非ホジキンリンパ腫
キメラ	IgG1κ	CD20	リツキシマブ	リツキサン	溶液	点滴静注	B 細胞性非ホジキンリンパ腫
キメラ	IgG1κ	EGFR	セツキシマブ	アービタックス	溶液	点滴静注	結腸・直腸がん
ヒト化	IgG1κ	VEGF	ベバシズマブ	アバスチン	溶液	点滴静注	結腸・直腸がん
ヒト化	IgG1κ	HER2	ペルツズマブ	パージェタ	溶液	点滴静注	乳がん
ヒト化	IgG1κ	HER2	トラスツズマブ	ハーセプチン	凍結乾燥	点滴静注	転移性乳がん
ヒト化	IgG1κ（糖鎖改変）	CCR4	モガムリズマブ	ポテリジオ	溶液	点滴静注	成人 T 細胞白血病リンパ腫
ヒト化	IgG4κ (カリケアマイシン修飾)	CD33	ゲムツズマブオゾガマイシン	マイロターグ	凍結乾燥	点滴静注	急性骨髄性白血病
ヒト	IgG1κ	CD20	オファツムマブ	アーゼラ	溶液	点滴静注	慢性リンパ性白血病
ヒト	IgG2κ	EGFR	パニツムマブ	ベクティビックス	溶液	点滴静注	結腸・直腸がん
免疫調節薬							
キメラ	IgG1κ	TNFα	インフリキシマブ	レミケード	凍結乾燥	点滴静注	関節リウマチ
キメラ	IgG1κ	CD25	バシリキシマブ	シムレクト	凍結乾燥	静脈内	腎移植後の急性拒絶反応抑制
ヒト化	IgG1κ	IL6R	トシリズマブ	アクテムラ	溶液	点滴静注, 皮下	関節リウマチ
ヒト化	IgG1κ	IgE	オマリズマブ	ゾレア	凍結乾燥	皮下	気管支喘息
ヒト化	IgG1κ	RS ウイルス	パリビズマブ	シナジス	凍結乾燥, 溶液	筋肉内	RS ウイルス感染
ヒト化	Fab′ (PEG 化低分子抗体)	TNF 抗体	セルトリズマブ ペゴル	シムジア	溶液	皮下	関節リウマチ
ヒト	IgG1κ	TNFα	アダリムマブ	ヒュミラ	溶液	皮下	関節リウマチ
ヒト	IgG1κ	IL12/IL23-p40	ウステキヌマブ	ステラーラ	溶液	皮下	尋常性乾癬
ヒト	IgG1κ	TNFα	ゴリムマブ	シンポニー	溶液	皮下	関節リウマチ
ヒト	IgG1κ	IL-1β	カナキヌマブ	イラリス	凍結乾燥	皮下	クリオピリン関連周期性症候群
ヒト	IgG2/4κ	補体 C5	エクリズマブ	ソリリス	溶液	点滴静注	発作性夜間ヘモグロビン尿症
その他							
ヒト化	Fab（低分子抗体）	VEGF	ラニビズマブ	ルセンティス	溶液	硝子体内	加齢黄斑変性症
ヒト	IgG2	RANKL	デノスマブ	ランマーク, プラリア	溶液	皮下	骨病変，骨粗鬆症

一般名の（遺伝子組換え）は省略して表記した.



（参考文献：Klein JS et al. JBC 106, 7385, 2009; Sarin H, J Angiogenesis Res 2, 13, 2010, Bagby TR et al. Pharmaceutics 4, 276, 2012, Moeller MJ and Tenten V Nat. Rev. Nephrol. 9, 266, 2013）

図 4　抗体医薬品の分子量・分子サイズと，細胞間隙経路の大きさ

免疫調節薬では皮下投与が多い（表 1）．免疫調節薬が用いられる慢性疾患では，自己注射が可能な皮下投与製剤が好まれる傾向が強くなっており，静脈内投与製剤が承認されて数年後に，皮下投与製剤が開発・承認される例も出てきている．言うまでもなく，皮下投与製剤では液量が限られ，高濃度の溶液が必要となる．抗体医薬品の投与量は高く，数十 mg/mL 程度の高濃度の溶液が必要になることもあり，製剤処方の最適化のみでは目的とする濃度での溶液製剤の作製が困難な場合もあり得るため，分子設計の段階から，製剤化を考慮した分子の選択が必要になる．

3.2.2　安定性

抗体医薬品製剤は，溶液製剤または凍結乾燥製剤で，通例，4°C で保存される．有効期間の設定には，実時間実保存条件での安定性試験が必要であり，最終的な評価には長時間を要する．安定性の評価項目は，有効性・安全性に影響する品質特性となるが，製剤の保存中にもその含量が増加し得る凝集体は，免疫原性等，安全性への影響が懸念される不純物であるので，製剤の安定性を考える上で，特に注意が必要とされる．また，脱アミドや酸化等の化学的な修飾，高次構造変化等も有効性・安全性に影響する品質特性として，安定性の評価項目になる可能性が高い．安定性試験結果で問題が生じる事態を避けるため，分子設計の段階で，凝集体形成や化学修飾の懸念が少ないアミノ酸配列を選択することが望ましい．凝集体形成を起こしやすいアミノ酸配列を予測する方法が検討されており[5]，これらに一致する配列を回避する等の分子設計が考えられる．

3.3　製造工程

IgG 骨格を持つ典型的な抗体医薬品の製造工程としては，プラットフォーム化された技術があり，分子設計の際に考慮すべきことは多くない．しかし，その他では，個別に対応すべき問題を考慮して，分子設計を行う必要がある．代表的な例は二重特異性抗体である．抗原 A に結合する H 鎖，L 鎖，抗原 B に結合する H 鎖，L 鎖からは，10 通りの分子種が生じ得るため，目的とする抗体の収率は低い．これを回避するため，2 種類の H 鎖にそれぞれ鍵と鍵穴となるアミノ酸置換を施し，目的とする H 鎖の会合を促進する方法や，L 鎖の共通化等の分子設計が行われている．また，Fc ドメインを持たない低分子抗体の精製には，IgG 型抗体で汎用される Protein A カラムを用いることができないため，別のアフィニティーカラムに結合させるためのアミノ酸配列が導入される例もある．培養上清中の安定性が悪い等，大量生産に適さない抗体は，除外すべきである．

4.　抗体医薬品の体内動態制御のための分子設計

抗体医薬品の体内動態には，有効成分の分子サイズ，荷電，及び，FcRn 結合性[6]等が関与する．図 4 に，抗体医薬品の分子サイズと，組織毛細血管，リンパ管，及び，腎糸球体基底膜の細胞間隙の大きさを示した．皮下投与の際，低分子抗体は，毛細血管

及びリンパ管から吸収される大きさで，IgG型抗体はリンパ管から吸収される大きさである．低分子抗体は，糸球体ろ過を受けるサイズであるため，消失には，糸球体ろ過の寄与が大きい．IgG型抗体は，糸球体ろ過されず，その消失には，細胞への非特異的取り込みに伴う分解，及び，標的介在性の薬物消失（Target mediated drug disposition），さらに，これらに対するFcRnの抑制効果が関与する（図1B-ii）．

4.1 IgG型抗体

IgG型の抗体医薬品では，他のタンパク質医薬品と比較して血中滞留性はよいが，標的介在性の薬物消失等により，血中半減期が2〜5日程度のものもある．また，一度，抗原に結合した抗体医薬品は，リサイクルされても，再度別の抗原に結合することはできず，生体内に存在しても機能を発揮できない．

これらの課題を踏まえ，血中半減期の延長や，抗原の結合していない遊離型抗体をリサイクルさせるための分子設計が行われている．血中半減期の延長には，Fcドメインのアミノ酸置換により，FcRn結合親和性を上昇させる分子設計が行われており，動物実験では，野生型の4倍程度までの血中半減期の延長がみられている[7]．遊離型抗体のリサイクリングには，細胞内エンドソームの酸性条件下で荷電状態が変わるHis残基をCDRに導入する手法が開発されており，この手法を用いると，抗原抗体複合体が細胞内に取り込まれた後，エンドソーム内で抗原が抗体から解離するため，遊離型抗体のみがFcRnによりリサイクルされる[8]．これらの分子設計による体内動態改良は，投与量や投与頻度の低減につながり，皮下投与製剤の開発可能性も高めるものと考えられる．

4.2 低分子抗体

IgG型の抗体医薬品とは対照的に，Fcドメインを持たない低分子抗体医薬品は，FcRnによるリサイクリングを受けず，また，糸球体ろ過により消失するため，半減期が数時間程度と短い[9]．血中半減期を延長し，有効血中濃度を維持するための分子設計として，Fcドメイン，あるいは，IgGに結合するペプチドと低分子抗体を融合することで，直接あるいは間接的にFcRn結合性を付与する試みがなされている[7]．また，FcRnには，IgGのみならず，アルブミンも結合するため，Fcに代わり，アルブミンを利用することも試みられている（図2）．

4.2.1 直接的FcRn結合性付与

低分子抗体-Fc融合タンパク質として，scFv，あるいは，二重特異性scFvとFcの融合タンパク質等の作製が報告されており，scFvでは3.5時間であった血中半減期が，scFv-Fcでは93時間に延長された例もある[10]．また，低分子抗体-アルブミン融合タンパク質として，scFv，あるいは，single chain diabodyとアルブミンの融合タンパク質，また，diabodyとアルブミンドメインIII融合タンパク質等の分子設計の例がある．

4.2.2 間接的FcRn結合性付与

IgG結合配列として，IgG結合性を持つタンパク質であるProtein A，Protein G，あるいはProtein L由来のペプチド配列を低分子抗体に付与する方法が開発されており，低分子抗体-IgG結合性ペプチド融合タンパク質として，scFv，あるいは，single chain diabodyとIgG結合性ペプチドの融合タンパク質等が報告されている．IgG上のProtein A及びProtein G結合部位は，FcRn結合部位に近いため，IgGのFcRnへの結合を阻害しないペプチド配列を選択することが重要となる．ペプチドを利用する方法では，Fcドメインやアルブミンとの融合タンパク質とする場合と比べ，分子量が大きくならないため，組織浸透性を保てる可能性が高くなると考えられる．

4.2.3 その他

他のバイオ医薬品の血中半減期延長のために用いられているPEG化は，低分子抗体医薬品にも応用されており，PEG化されたFab構造を持つセルトリズマブ ペゴルがその例である（表1）．有効成分の構造を改変する手法の他，開発中の製品では，ポンプを用いて低分子抗体を持続注入する方法も用いられており，投与デバイスの工夫で有効血中濃度を維持する手法も含め，体内動態の制御には，様々な手法が考えられる．

5. おわりに

抗体医薬品の分子設計において考慮すべきポイントについて，薬理，薬物動態，免疫原性，溶解性，安定性，製造工程の観点から考察した．抗体医薬品の分子設計は，生産用細胞株の樹立，治験薬製造，非臨床試験，臨床試験等からなる一連の開発過程の入り口である．アミノ酸配列が一つでも異なれば別

の医薬品となるため，開発途中での構造改変は，その抗体医薬品の開発中止を意味する．実生産スケールでの製造や，ヒトでの有効性・安全性等，開発の後期にならないと分からないことも多い中，最適と思われる構造を選択していかなければならない．タンパク質工学を中心に，開発に関わる様々な分野の連携が重要と思われる．

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Polyunsaturated fatty acid saturation by gut lactic acid bacteria affecting host lipid composition

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Edited by Jerrold Meinwald, Cornell University, Ithaca, NY, and approved September 10, 2013 (received for review July 11, 2013)

In the representative gut bacterium *Lactobacillus plantarum*, we identified genes encoding the enzymes involved in a saturation metabolism of polyunsaturated fatty acids and revealed in detail the metabolic pathway that generates hydroxy fatty acids, oxo fatty acids, conjugated fatty acids, and partially saturated *trans*-fatty acids as intermediates. Furthermore, we observed these intermediates, especially hydroxy fatty acids, in host organs. Levels of hydroxy fatty acids were much higher in specific pathogen-free mice than in germ-free mice, indicating that these fatty acids are generated through polyunsaturated fatty acids metabolism of gastrointestinal microorganisms. These findings suggested that lipid metabolism by gastrointestinal microbes affects the health of the host by modifying fatty acid composition.

biohydrogenation | hydratase | fatty acid isomerase | conjugated linoleic acid | lipid nutrition

Dietary fats are metabolized not only by humans but also by microbes in our gastrointestinal tracts. Microorganisms in the gastrointestinal tract interact with their host in many ways and contribute significantly to the maintenance of host health (1). Lipid metabolism by gastrointestinal microbes generates multiple fatty acid species, such as conjugated fatty acids and *trans*-fatty acids, that can affect host lipid metabolism (2). However, lipid metabolism by gastrointestinal microbes has not been explored in detail. Saturation metabolism of polyunsaturated fatty acids, a representative mode of lipid metabolism by gastrointestinal microbes, is a detoxifying metabolism of anaerobic bacteria, such as lactic acid bacteria, that reside in colon and intestine. This process transforms growth-inhibiting free polyunsaturated fatty acids into less toxic free saturated fatty acids (3). This saturation metabolism generates characteristic fatty acids (e.g., conjugated fatty acids and *trans*-fatty acids, which are well known to present in ruminant-derived foods and exert various physiological activities).

"Conjugated fatty acid" is a collective term for positional and geometric isomers of fatty acids with conjugated double bonds. In particular, conjugated linoleic acids (CLAs), such as *cis*-9,*trans*-11-CLA and *trans*-10,*cis*-12-CLA, reduce carcinogenesis (4), atherosclerosis (5), and body fat (6). With regard to lipid metabolism, CLA is a potent peroxisome proliferator-activated receptor (PPAR)α agonist (7), and treatment with CLA increases the catabolism of lipids in the liver of rodents (8). Based on these findings, CLA is now commercialized as a functional food for control of body weight, especially in the United States and European countries.

On the other hand, consumption of *trans*-fatty acids increases the risk of coronary heart disease by increasing LDL and reducing HDL cholesterol levels (9). Consequently, *trans*-fatty acids are considered to be harmful for health, and nutritional authorities have recommended that consumption of *trans*-fatty acids be reduced to trace amounts (10). Therefore, it is important to control fatty acid saturation processes that generate these fatty acids (11); however, the precise metabolic pathway and enzymes involved have not been clearly identified.

Our analyses on conjugated fatty acid synthesis in representative gut bacteria, the lactic acid bacteria (12–15), demonstrated that *Lactobacillus plantarum* AKU 1009a (AKU Culture Collection, Faculty of Agriculture, Kyoto University) can transform the *cis*-9,*cis*-12 diene structure of C18 fatty acids such as linoleic acid, α-linolenic acid, and γ-linolenic acid into the conjugated diene structures *cis*-9,*trans*-11 and *trans*-9,*trans*-11 (16–21). In addition, this strain can saturate these conjugated dienes into the *trans*-10 monoene. Our subsequent metabolic analysis indicated that 10-hydroxy-12-octadecenoic acid is an intermediate of CLA synthesis, and further investigations of hydroxy fatty acid metabolism by lactic acid bacteria revealed that CLA is produced from hydroxy fatty acids such as ricinoleic acid in castor oil (22–25). In cell-free extracts from this strain, we identified the enzymes involved in CLA synthesis (26). Three enzymes, CLA-HY, CLA-DH, and CLA-DC, are necessary for synthesis of conjugated fatty acids such as CLA. Only the combined action of these three enzymes can generate CLA from linoleic acid, with 10-hydroxy-*cis*-12-octadecenoic acid arising as an intermediate

Significance

Microorganisms in the gastrointestinal tract interact with their host in many ways. Lipid metabolism by gastrointestinal microbes generates multiple fatty acid species that can affect host health. In the representative gut bacterium *Lactobacillus plantarum*, we revealed a fatty acid metabolism, saturation metabolism of polyunsaturated fatty acid, that generates hydroxy fatty acids, oxo fatty acids, conjugated fatty acids, and partially saturated *trans*-fatty acids as intermediates. Furthermore, fatty acid analysis in mice suggests that the fatty acid metabolism by gastrointestinal microbes modifies fatty acid composition of the host. Therefore, functional investigations of lipid metabolisms of gastrointestinal microbes may provide new methods for improving our health by altering lipid metabolism related to the onset of metabolic syndrome.

Author contributions: S.K., S.S., and J.O. designed research; S.K., M.T., S.-B.P., A.H., N.K., J.K., R.I., Y.I., and M.A. performed research; S.K., S.-B.P., J.K., H.K., M.A., H.A., K.U., J.S., S.T., K.Y., and S.S. analyzed data; and S.K., M.T., and J.O. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AB812091).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1312937110/-/DCSupplemental.

(Fig. 1*B*, *3*). The reactions catalyzed by each enzyme, however, were not revealed in those studies. Through genomic analysis in *L. plantarum* WCFS1, we found that *cla-dh* (GenBank accession no. NC_004567; region: 59613-60473) and *cla-dc* (GenBank accession no. NC_004567; region: 60505-61350) are located in a cluster with another gene, *cla-er* (GenBank accession no. NC_004567; region: 61378-62031) (Fig. 1*A*). In light of this, we tried to identify the function of the gene product (CLA-ER) together with those of CLA-HY, CLA-DH, and CLA-DC.

Results and Discussion

Based on the sequence of the *L. plantarum* WCFS1 genome, we designed primers to amplify the CLA-ER gene from *L. plantarum* AKU 1009a genomic DNA. The PCR-amplified product, *cla-er* (GenBank accession no. AB812091), consisted of 654 bp and encoded a protein whose amino acid sequence is 99.8% identical to the homologous sequence from *L. plantarum* WCFS1. We transformed the resulting plasmid containing *cla-er* from *L. plantarum* AKU 1009a, pCLA-ER, into *Escherichia coli* Rosetta2 (DE3) to generate *E. coli* Rosetta/pCLA-ER. The gene product, CLA-ER, had a molecular mass of ~25 kDa (including a His tag) and could be detected in soluble cell-free extracts of *E. coli* Rosetta/pCLA-ER. We examined the function of CLA-ER in fatty acid metabolism using purified CLA-ER in combination with other purified enzymes (CLA-HY, CLA-DH, and CLA-DC) from the corresponding transformants (*E. coli* Rosetta/pCLA-HY, *E. coli* Rosetta/pCLA-DH, and *E. coli* Rosetta/pCLA-DC).

In a reaction with these four enzymes (CLA-HY, CLA-DH, CLA-DC, and CLA-ER) as catalysts, *cis*-9,*trans*-11-CLA (CLA1), *trans*-9,*trans*-11-CLA (CLA2), 10-hydroxy-*cis*-12-octadecenoic acid,

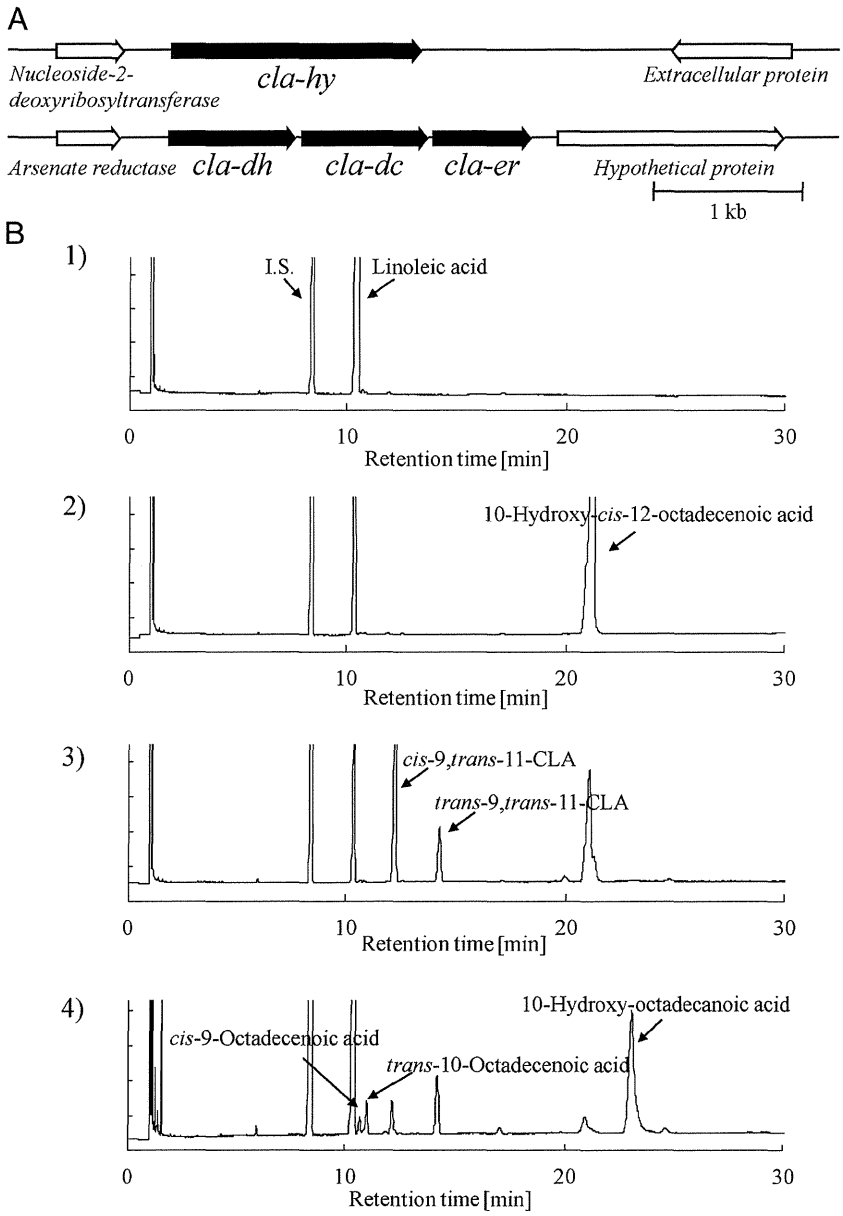

Fig. 1. Gene clusters for polyunsaturated fatty acid metabolism and GC chromatograms. (*A*) Gene clusters for fatty acid metabolic enzymes in *L. plantarum*. (*B*) GC chromatograms of substrate (*1*); reaction with CLA-HY (*2*); reaction with CLA-HY, CLA-DH, and CLA-DC together with FAD and NADH (*3*); and reaction with CLA-HY, CLA-DH, CLA-DC, and CLA-ER together with FAD and NADH (*4*). I.S., internal standard.