研究報告書

厚生労働科学研究費補助金（再生医療実用化研究事業）
分担研究報告書

人工多能性幹細胞の品質・安全性に関する研究

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研究要旨：細胞組織加工医薬品に供する細胞資源として、人工多能性幹細胞（iPS細胞）の期待が集まっている。iPS細胞は、容易に自己由来多能性幹細胞をもたらす画期的細胞制御技術であるが、現段階では樹立・作製法の改良はもとより、日本人由来iPS細胞の特性把握が必要である。本分担研究では、特に細胞の同一性・同等性評価法の開発、特性解析法の開発を行った。様々な年齢の日本人の線維芽細胞から樹立したiPS細胞は、形態学的特、ES細胞マーカー、細胞表面抗原、in vitroおよびin vivoの分化能の点で、ほぼ同様であり、ES細胞に比肩するものであった。また、ヒトiPS細胞の細胞表面抗原に基づく細胞評価法や、DNAマイクロアレイによる特性解析法について、基盤的知見が得られた。

A．研究目的

細胞組織加工医薬品は、様々な難治性疾患に対して有効な治療法をもたらすと期待される一方、未知・未経験な要素が多く本格的な実用化に至るために検討すべき課題はまだ数多い。細胞組織加工医薬品は、非常に複雑な構造と「生きている」とうこれまでの医薬品にない性質から、新たな特性解析技術や品質管理法の開発、あるいは安全性確保のための適切な評価技術の開発が望まれている。数多くある細胞資源において、特に、2007年11月に本邦で世界に先駆けて樹立されたヒト体細胞由来の人工多能性幹細胞（iPS細胞）については、容易に自己由来多能性幹細胞をもたらす画期的技術である一方で、現段階では樹立・作製法の改良はもとより、日本人由来iPS細胞の特性把握が必要である。本分担研究ではiPS細胞をベースにした細胞組織加工医薬品の品質・安全性の確保を目的として、（1）ウイルス安全性確保を目的とした、核酸増幅法（NAT）によるウインドウ期や低濃度キャリアーの高感度ウイルススクリーニング法の開発およびNATのバリデーション手法の標準化に関する研究、（2）細胞表現型プロファイリングによる細胞の同一性・同等性評価法の開発、（3）細胞組織加工医薬品の特性解析法の開発および製造方法・規格設定の評価手法の開発に関する研究を行う。これらの研究によって、iPS細胞に立脚した将来の細胞組織加工医薬品に発展させる知見をもたらし、安全性確保や品質管理法の開発に寄与出来るものと期待される。

B．研究方法

1）高感度ウイルススクリーニング法や感染性因子の迅速法の開発

感染性因子に関する安全性確保の一環と

して、ポリエチレン磁気ビーズを用いたヒト肝炎ウイルス等の濃縮法等と核酸増幅法（NAT）を組み合わせることにより、ウインドウ期や低濃度キャリアーの高感度ウイルススクリーニング法の開発を行う。
また、無菌試験の迅速法の基盤技術開発を行う。これらに成果に基づいて日米欧のウイルス検出のためのNATガイドラインによるバリデーション手法への適用やマイコプラズマへの応用ついて検討を行う。

2）細胞の同一性・同等性評価法の開発

細胞の培養、増幅、加工過程を通じての細胞の恒常性や、目的とする細胞への分化誘導による特性の付与を、細胞表面糖鎖の恒常性・変化から適切に捉えるために、網羅的な全細胞糖鎖の解析法を開発する。同時に、開発した糖鎖プロファイリング技術を細胞特性解析、がん化予測法開発、製造工程由来不純物検出法開発等への応用に関する研究を行う。

3）特性解析法の開発

細胞治療薬は有効性・安全性に関わる品質特性の評価法開発の一環として、ヒト血管内皮前駆細胞やヒト間葉系幹細胞、さらにはiPS細胞由来機能性細胞を用いて、その品質を評価するための特性指標を探索する。DNAマイクロアレイ、プロテインアレイ等を利用した細胞特性指標探索法としての有用性を評価する。開発した小型マイクロアレイを含め特性指標探索法の標準化に必要な要素を明らかにし、指針等への反映を目指す。

（倫理面への配慮）
iPS細胞のソースとして、日本人線維芽細胞は適切な手続きをとって公的リソースバンクなどから入手使用した。実験動物に対しては、無用な苦痛を与えずに細胞投与などの処置を行い、検体採取時には安楽死させ、無用な苦痛を与えないようガイドラインに従って取り扱った。

C．研究結果

1）高感度ウイルススクリーニング法や感染性因子の迅速法の開発

担当する研究３課題のうち、他２課題を優先させており、本研究の成果は次年度以降、報告を行う。

2）細胞の同一性・同等性評価法の開発

これまで分担者が報告したヒトiPS細胞は白人由来のものであり、本邦にてiPS細胞に立脚した将来の細胞組織加工医薬品に発展させるには、日本人由来iPS細胞に関する知見が必要である。そこで、６歳から81歳の男女に由来する線維芽細胞を入手し、レトロウイルスベクターによりOct3/4、Sox2、Klf4、c-Mycを導入し、 iPS細胞を樹立した。これらのiPS細胞のコロニーは典型的なヒトES細胞様の形態を示した。RT-PCRにより、ES細胞マーカーの発現は、ヒトES細胞や、私達の既報の白人由来iPS細胞と同様であった。本研究項目の解析対象である細胞表面抗原について、全日本人iPS細胞について、SSEA-1、SSEA-3、およびTRA-1-81などに対する抗体で染色を行ったところ、それらの結果は、ES細胞と同様であることが明らかとなった。継代を行っていっても、これらの特徴が同様に保持されるのか詳細に調査する。また、いくつかの年齢の異なるiPS細胞について胚葉体形成法によるin vitro分化能や、SCIDマウス移植によるテラ

トーマ形成法でin vivo分化能を調べたが、多能性の観点で年齢差は認められなかった。

3）特性解析法の開発

上述の日本人 iPS 細胞を用いて品質特性の評価法開発の一環として、まず DNA マイクロアレイによる遺伝子発現の網羅的解析を行った。Cyanine 3 で標識したトータル RNA を調製し、全ヒトゲノムマイクロアレイにハイブリダイズさせ、解析データ解析をヒートマップなどにまとめた。その結果、線維芽細胞と iPS 細胞は非常に遺伝子発現が異なることが明らかとなった。一方、iPS 細胞間および iPS 細胞と ES 細胞間の違いは極めて小さかった。今後、iPS 細胞を分化誘導させて得られる様々な系譜の細胞について同様の解析を行っていき、細胞機能との相関を調べ、DNA マイクロアレイの細胞評価における有効性を検証していく予定である。

D．考察

iPS細胞は、個人の年齢や性別にかかわらず樹立が可能であった。樹立された日本人iPS細胞は、形態学的特徴、ES細胞マーカー、細胞表面抗原、in vitroおよびin vivoの分化能の点で、ほぼ同様であり、ES細胞に比肩するものであった。今回は、樹立直後の細胞の解析であったが、継代を重ねていった場合、核型も含めて細胞表面抗原や網羅的遺伝子発現状況などに変動がないか研究を行っていく。

E．結論

iPS細胞は、体細胞をES細胞に近似させる細胞制御技術である。今回、様々な年齢の日本人からもiPS細胞の樹立は可能であり、それらの特性差は極めて小さいことが確認された。本邦におけるiPS細胞に立脚した細胞組織加工医薬品の開発に資する基盤的知見が得られた。

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別紙４

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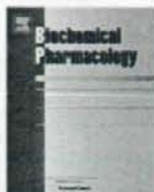

The RXR agonists PA024 and HX630 have different abilities to activate LXR/RXR and to induce ABCA1 expression in macrophage cell lines

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ABSTRACT

Release of cellular cholesterol by ATP-binding cassette transporter (ABC)A1 and apolipoproteins is a major source of plasma high-density lipoprotein (HDL). Expression of ABC transporter A1 (ABCA1) is directly stimulated by liver X receptor (LXR)/retinoid X receptor (RXR) activation. We evaluated the abilities of two RXR agonists, PA024 and HX630, to increase ABCA1 expression. In differentiated THP-1 cells, the two agonists efficiently enhanced ABCA1 mRNA expression and apoA-I-dependent cellular cholesterol release. However, in RAW264 cells and undifferentiated THP-1 cells, PA024 was highly effective while HX630 was inactive in increasing ABCA1 mRNA. In parallel, the two agonists had different abilities to activate ABCA1 promoter in an LXR-responsive-element (LXRE)-dependent manner and to directly stimulate LXRα/RXR transactivation. The ability of HX630 to enhance ABCA1 expression was correlated closely with the cellular PPARγ mRNA level. Moreover, HX630 was able to activate PPARγ/RXR. Transfection of PPARγ in RAW264 cells induced HX630-mediated activation of LXRE-dependent transcription and ABCA1 promoter, suggesting the ability of HX630 to activate PPARγ-LXR-ABCA1 pathway. We conclude that RXR agonist PA024 and HX630 have different abilities to activate LXR/RXR, and that the cell-type-dependent effect of HX630 on ABCA1 expression and HDL generation is closely associated with this defect.

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1. Introduction

ABC transporter A1 (ABCA1) mediates and rate-limits biogenesis of high-density lipoprotein (HDL) from helical apolipoprotein acceptors, such as apoA-I, and cellular cholesterol and phospholipids [1]. Mutations in the ABCA1 gene cause Tangier disease and other genetic HDL deficiencies [2–4]. Conversely, overexpression of ABCA1 in mice resulted in a mild elevation

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of HDL cholesterol [5,6] and has been shown to protect animals from atherosclerosis [7,8]. Plasma HDL levels are inversely related to the risk of atherosclerotic cardiovascular disease [9], presumably because HDL functions to remove excess cholesterol from peripheral tissues and transport it to the liver for conversion to bile acids [10]. Accordingly, therapies that increase ABCA1 expression are a promising strategy for preventing and treating atherogenesis.

Cellular expression of ABCA1 is highly regulated. Loading cholesterol into macrophages and fibroblasts resulted in enhanced transcription of the ABCA1 gene by the reaction mediated by the oxysterol-activated liver X receptor (LXR) [11–14]. ABCA1 expression can also be increased by PPARα or PPARγ activators [15].

Retinoid X receptor (RXR) is a member of the nuclear receptor superfamily and forms heterodimers with a number of other receptors. RXR heterodimers, such as the peroxisome proliferator-activated receptor (PPAR)/RXR, LXR/RXR and the farnesoid X receptor (FXR)/RXR, can be activated by agonists for both RXR and the partner receptors and are classified as permissive heterodimers [16–18]. The thyroid hormone receptor/RXR or vitamin D receptor/RXR are not activated by RXR agonists and termed as non-permissive heterodimers [16–18]. A natural RXR agonist, 9-cis-retinoic acid, and synthetic RXR-selective ligands (rexinoids) have been shown to increase ABCA1 expression in macrophages [12,19,20].

In the present study, we evaluated the ability of two RXR agonists, PA024 and HX630, to induce ABCA1 expression in macrophage cell lines. Both PA024 and HX630 have been developed as RXR-selective agonists and are inactive alone in the HL-60 differentiation assay but strongly enhance the activity of low concentration of RAR-selective agonist AM80 [21]. However, their effects on the other RXR heterodimers are unknown. We found that PA024 potently induces ABCA1 expression in all cell models examined. However, HX630 failed to induce ABCA1 expression in RAW264 cells and undifferentiated THP-1 cells, and this defect was closely associated with the lack of ability to activate LXR/RXR. Instead, HX630 was able to activate PPARγ/RXR and induce ABCA1 expression in differentiated THP-1 cells. Our data also suggest the ability of HX630 to stimulate the PPARγ-LXR-ABCA1 pathway.

2. Materials and methods

2.1. *Materials*

22(R)-hydroxycholesterol and phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma; troglitazone from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA, USA) HX630, PA024, and Am80 were prepared as described previously [21–23].

2.2. *Cell culture and real time quantitative RT-PCRs*

RAW264 cells were obtained from the Riken Gene Bank (Tsukuba, Japan) and maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 (1:1) containing 10% fetal calf serum. Cells were incubated for 24 h in serum-free medium containing 0.1% BSA in the presence or absence of 22(R)-hydroxycholesterol (2 μg/ml) and RXR agonists. THP-1 cells were maintained in RPMI 1640 medium containing 10% fetal calf serum. Cells were treated with RXR agonists in serum-free medium containing 0.2% BSA. Differentiation of THP-1 cells into macrophages was induced by treatment of the cells with 100 nM PMA for 48 h. RXR agonists were added to the medium during the last 24 h. Cells were harvested and total RNA was extracted using an RNeasy Mini Kit (Qiagen). The RNA samples were treated with DNase according to the manufacturer's protocol (Qiagen). Relative expression levels of mRNA were determined using a TaqMan one-step RT-PCR Master Mix Reagent Kit and an ABI Prism 7700 sequence detection system (Applied Biosystems). Primer/probe sequences used were as follows: human PPARγ forward primer, 5′-AGGCGAGGGCGATCTTG-3′, reverse primer, 5′-CCCATCATTAAGGAATTCATGTCAT-3′, probe, 5′FAM-CAGGAAAGACAACAGACAAATCACCATTCGT-TAMRA3′. The primer/probe sequences for mouse ABCA1 [24], human ABCA1, human ABCG1, and human LXRα [25] were the same as described previously. Expression data were normalized to 18S rRNA levels, and presented as the fold difference of treated cells against untreated cells.

2.3. *Plasmid constructs*

Plasmids for a LXRE-driven luciferase reporter and a PPAR response element (PPRE)-driven luciferase reporter (pPPRE-tk-Luc) and were constructed by inserting the cDNAs containing two copies of LXREa and LXREb from the sterol response element binding protein-1c promoter and two copies of PPRE from acyl-CoA oxidase, respectively, upstream from the thymidine kinase (tk) promoter. cDNAs encoding full-length human RXRα, LXRα, LXRβ, or PPARγ were PCR-cloned and inserted into mammalian expression vector pcDNA3.1 (Invitrogen). A mouse peripheral-type ABCA1 promoter (−1238/+57 of exon 1)-luciferase vector (pABCA1-Luc) has been described previously [24]. A mouse ABCA1 promoter construct containing mutation in LXRE (pABCA1:mutLXRE-Luc) was prepared as described previously [26].

2.4. *Transient transfections and reporter gene assays*

RAW264 cells were transfected with 1.0 μg of pABCA1-Luc or pABCA1:mutLXRE-Luc and 0.1 μg of Renilla luciferase vector (phRL-TK) (Promega) with SuperFect (Qiagen) in 24-well plates. For LXR activation studies, 1 μg of pLXRE-tk-Luc, 50 ng each of pcDNA3.1-LXR and pcDNA3.1-RXRα, and 0.7 μg of pSV-β-galactosidase control vector (Promega) were used. For the assay of PPARγ activation, cells were transfected with 1.3 μg of pPPRE-tk-Luc and 0.1 μg of Renilla luciferase vector (phRL-TK) (Promega) in the presence or absence of 50 ng each of pcDNA3.1-LXR and pcDNA3.1-RXRα. An empty pcDNA3.1 expression vector was used to maintain equal amounts of DNA for each transfection. Three hours after transfection, cells were exposed to RXR agonists in the medium containing 10% FCS for 24 h. Undifferentiated THP-1 cells were electroporatically transfected with 0. 4 μg of pABCA1-Luc or empty vector and 0.1 μg of phRL-TK using the Nucleofector transfection system (Amaxa Inc., Gaithersburg, MD) according to the manufacturer's protocol. Four hours after transfection, cells were exposed to RXR agonists in the medium containing 0.2%

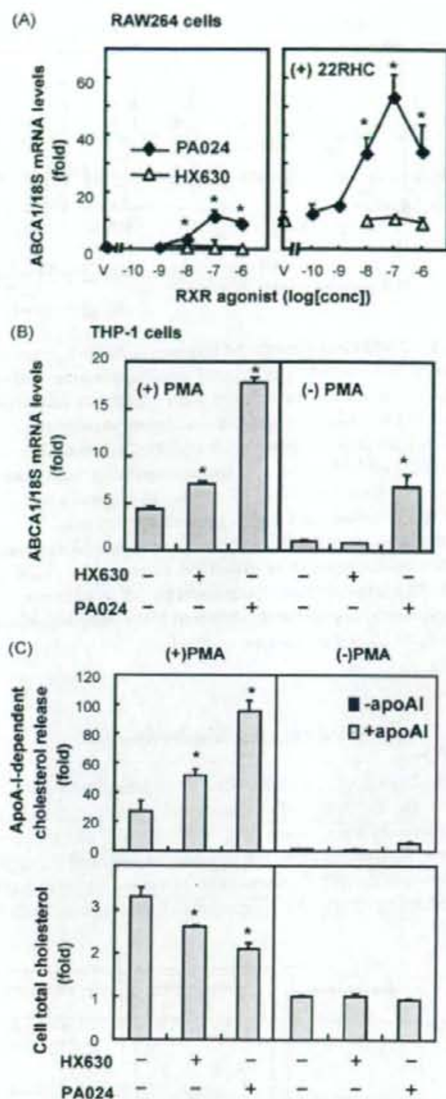

Fig. 1 – Effects of PA024 and HX630 on ABCA1 mRNA expression and cholesterol efflux in RAW264 cells, PMA-differentiated, and undifferentiated THP-1 cells. (A) RAW264 cells were treated for 24 h with PA024 or HX630 in the absence or presence of 22(R)-hydroxycholesterol (22RHC, 2 μg/ml). ABCA1 mRNA levels were measured with quantitative real-time RT-PCR analysis, standardized against 18S rRNA levels, and expressed as fold induction relative to the vehicle-treated cells (V, taken as 1). (B) PMA-differentiated or undifferentiated THP-1 cells were treated for 24 h with 100 nM PA024 or HX630. ABCA1 mRNA/18S rRNA levels were expressed as fold induction relative to the vehicle-treated undifferentiated cells. (C) PA024 and HX630 enhance apoA-I mediated cholesterol efflux and decrease the cellular cholesterol level in differentiated THP-1 cells. PMA-differentiated or undifferentiated THP-1 cells were treated for 24 h with 100 nM PA024 or HX630 in the presence or absence of apoA-I (15 μg/ml). ApoA-I-dependent release of cholesterol into the medium and cellular total cholesterol (2.69 ± 0.77 μg/mg protein and 15.31 ± 0.39 μg/mg protein, respectively, in control cells) were expressed as fold induction relative to the vehicle-treated undifferentiated cells. The values represent the average ± S.D. from three experiments. Significantly different from vehicle-treated cells (*).

FCS for 24 h. Luciferase and β-galactosidase activities were determined in cell lysates. Firefly luciferase activity was normalized to either that of Renilla luciferase or β-galactosidase for each well.

2.5. *Coactivator association assay using fluorescence polarization*

The assay was performed as described previously [27]. Briefly, TAMRA-labeled peptide (100 nM, with amino acid sequence ILRKLLQE) was incubated for 1 h with purified GST-fused human PPARγ LBD (1.5 μM) and ligands in 100 μl of buffer (10 mM Hepes, 150 mM NaCl, 2 mM $MgCl_2$, 5 mM DTT at pH 7.9) in a black polypropylene 96-well plate on a shaker. Ligand-dependent recruitment of the coactivator peptide was measured as increases in fluorescence polarization with a Fusionα-FP (PerkinElmer Life Science).

2.6. *Measurement of lipid efflux to apolipoprotein A-I (apoA-I)*

The differentiated or undifferentiated THP-1 cells were incubated in the presence or absence of 10 μg/ml of apoA-I in RPMI 1640 containing 0.2% BSA for 24 h. Lipid was extracted from the medium and the cells with chloroform/methanol (2:1, v/v) and hexane/isopropanol (3:2, v/v), respectively, and cholesterol and choline-phospholipid were determined by enzymatic methods specific for each lipid [28].

2.7. *Statistical analysis*

Data were analyzed by ANOVA followed by the Student–Newman–Keuls method. Statistical significance was established at the $P < 0.05$ level.

3. Results

3.1. *RXR agonist PA024 and HX630 show different abilities to induce ABCA1 expression*

We tested the ability of RXR agonist PA024 and HX630 to induce ABCA1 expression in murine macrophage-like cell line, RAW264. Treatment of RAW264 cells with PA024 markedly induced ABCA1 mRNA expression, which peaked at 100 nM, whereas HX630 up to 1 μM had no effect (Fig. 1A left). Expression of ABCA1 can be stimulated by oxysterol-activated

LXR [11,13,14]. The addition of 22(R)-hydroxycholesterol to the medium increased ABCA1 expression and strongly enhanced the effect of PA024 (Fig. 1A right). This combination had a greater than additive effect, whereas HX630 again had no effect.

The effect of two RXR agonists on ABCA1 expression was also studied in a human monocytic leukemia cell line, THP-1 (Fig. 1B). PA024 (100 nM) increased the ABCA1 mRNA level both in PMA-differentiated and undifferentiated cells (by 4- and 7-fold, respectively). In contrast, HX630 (100 nM) did not affect the ABCA1 mRNA level in undifferentiated cells. However, the same concentration of HX630 did raise the ABCA1 mRNA level in PMA-differentiated cells by 1.6-fold.

ABCA1 has been shown to play a critical role in the assembly of HDL from apoA-I and cellular cholesterol and phospholipids. We examined the effect of the two RXR agonists on apoA-I-mediated cholesterol release (HDL production) in THP-1 cells (Fig. 1C). HX630 and PA024 at 100 nM increased the amount of cholesterol released into the medium in the presence of apoA-I (by 2- and 3.4-fold, respectively) in differentiated cells, and this increase was accompanied by a decrease in the cellular total cholesterol level. In contrast, the same concentration of HX630 had no effect in undifferentiated cells.

3.2. *PA024 but not HX630 activates LXR/RXR and ABCA1 promoter*

To determine whether the different abilities of the two RXR agonists in ABCA1 mRNA induction were resulted from different abilities in ABCA1 gene transcription, we examined their effects on ABCA1 promoter activity. An ABCA1 promoter (−1238/+57 of exon 1)-luciferase construct was transfected into RAW264 cells. As shown in Fig. 2A, PA024 markedly increased the ABCA1 promoter transcription, and the increase caused by PA024 was lost when a mutation was introduced into LXRE in the promoter. In contrast, HX630 up to 1 μM had no effect on the promoter activity. Similarly, when the ABCA1 promoter was transfected into undifferentiated THP-1 cells, PA024 augmented the promoter activity in an LXRE-dependent manner, but HX630 had no effect (Fig. 2B). These findings indicate that PA024-induced activation of ABCA1 promoter was mediated by LXRE. We intended to examine the promoter activity in PMA-differentiated THP-1 cells. However, transfection of the plasmid into the differentiated THP-1 cells was unsuccessful. In addition, cells harboring transfected DNA did not undergo differentiation with PMA.

The possibility that PA024 but not HX630 activates LXR/RXR was tested using a reporter assay. Transfection of the LXRE-driven luciferase-reporter vector alone into RAW264 cells yielded substantial luciferase activity, indicating transcriptional activation by endogenous receptor(s), and this endogenous LXRs-mediated activity was increased by PA024, but not HX630 (Fig. 3 left). Co-transfection of LXRα and RXRα expression vectors enhanced the effect of PA024, whereas HX630 again had no effect (Fig. 3 middle). Expressions of LXRβ and RXRα augmented luciferase transcription in the vehicle-treated control (Fig. 3 right). However, the response elicited by PA024 was small and similar to that in cells without LXR/RXR plasmids (Fig. 3 left). HX630 again had no effect.

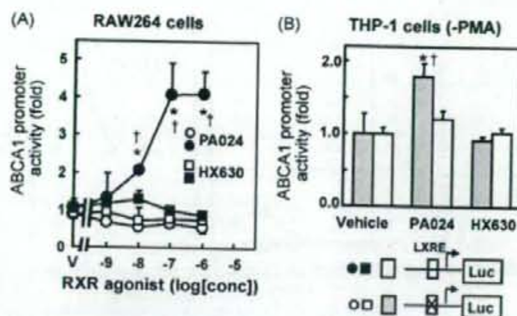

Fig. 2 – PA024 but not HX630 augments ABCA1 promoter activity in RAW264 cells (A) and undifferentiated THP-1 cells (B). Cells were transfected with a pABCA1-Luc (closed symbols) or pABCA1:mutLXRE-Luc (open symbols) reporter plasmid together with a phRL-TK internal control as described in Section 2.4, and treated with indicated concentrations (for A) or 100 nM (for B) of PA024 and HX630. Luciferase activity in the cell extract was normalized using Renilla luciferase activity and expressed as fold induction relative to vehicle-treated cells (indicated as V). The data represent the average ± S.D. of three experiments. Significantly different from vehicle-treated cells (*) or LXRE-mutated promoter (†).

3.3. *HX630 and PA024 activate PPARγ/RXR*

The ability of HX630 and PA024 to activate PPARγ/RXR was tested in RAW264 cells transfected with a PPRE-driven luciferase-reporter vector (Fig. 4A). When PPARγ/RXR was transfected into the cells, the two agonists markedly increased luciferase activity. RXR-homodimer has been shown to activate transcription of the DR-1 element [29]. Upon transfection of

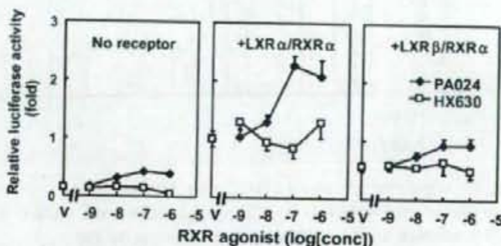

Fig. 3 – PA024 but not HX630 augments LXRα/RXR transactivation. RAW264 cells were transfected with LXREx4-tk-Luc reporter plasmid together with a β-gal internal control in the absence (A) or presence of expression plasmids for human LXRα and RXRα (B) or LXRβ and RXRα (C). The cells were treated with the indicated concentrations of PA024 or HX630. Luciferase activity in the cell extract was normalized using β-gal and expressed as fold induction relative to vehicle-treated cells (indicated as V). The data represent the average ± S.D. of three incubations.

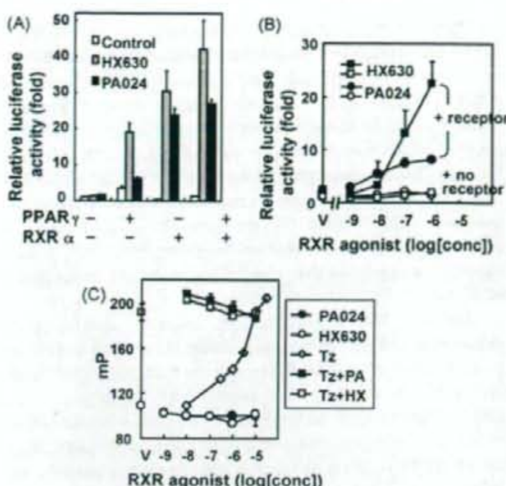

Fig. 4 – PA024 and HX630 stimulate PPARγ/RXR transactivation. (A) RAW264 cells were transfected with a PPRE-tk-Luc reporter plasmid together with a phRL-TK internal control in the presence or absence of expression plasmids for human PPARγ and RXRα. The cells were treated with 100 nM PA024 or HX630 for 24 h before analysis. (B) Dose–response of RXR agonists for activation of a reporter gene by PPARγ/RXR. Cells were transfected with a PPRE-tk-Luc reporter plasmid in the absence (open symbols) or presence (closed symbols) of the PPARγ expression plasmid and treated with drugs for 24 h. Luciferase activity stimulated by PPARγ and endogenous RXR in the cell extract was determined, normalized, and expressed as fold induction relative to vehicle-treated cells without exogenous receptor expression. The values represent the average ± S.D. of three incubations. (C) RXR agonists do not affect binding of troglitazone to PPARγ. A fluorescent-tagged SRC-1 peptide (0.1 μM) was incubated with 1.5 μM PPARγ ligand binding domain in the presence of various concentrations of PA024 and HX630 in the presence or absence of 10 μM troglitazone (Tz). Ligand-induced association of coactivator peptide with the receptor was monitored by increases in millipolarization fluorescence units (mP).

RXRα alone, the two agonists increased luciferase activity, indicating substantial activation of RXRα homodimer. However, when PPARγ alone was co-transfected, both agonists greatly enhanced transcription, suggesting that they have abilities to activate PPARγ/RXR heterodimer made from exogenous PPARγ and endogenous RXR. In this reporter assay system using exogenous PPARγ and endogenous RXR, HX630 activated PPARγ/RXR-dependent transcription more potently than PA024 at concentrations higher than 100 nM (Fig. 4B). To exclude the possibility that the HX630 and PA024 bind to PPARγ as a ligand, coactivator-association assay was performed. As shown in Fig. 4C, neither HX630 nor PA024 induced an interaction between fluorescence-labeled coactivator peptide and the PPARγ ligand binding domain *in vitro*, while the PPARγ agonist troglitazone strongly induced association. Furthermore, these RXR agonists did not affect the PPARγ-coactivator interaction elicited by 10 μM troglitazone. These findings indicate that the two RXR agonists were able to activate PPARγ/RXR.

3.4. *Possible stimulation of PPARγ-LXR-ABCA1 pathway by HX630*

LXRα gene is known to be a direct target of PPARγ/RXR, and stimulation of PPARγ has been shown to increase LXR-target gene expression by increasing LXRα expression [30]. We therefore examined the ability of HX630 to enhance LXR-target gene transcription by activating PPARγ/RXR. LXRE-driven luciferase assay was performed in RAW264 cells with or without co-transfection of PPARγ. As shown in Fig. 5A, HX630 augmented luciferase transcription in the presence but not in the absence or PPARγ. This effect was enhanced by increasing the amount of the PPARγ expression plasmid and the combination of HX630 and the LXR agonist 22(R)-hydroxycholesterol had an additive effect. The activity of ABCA1 promoter was also increased by HX630 when PPARγ was co-transfected (Fig. 5B)

The level of PPARγ mRNA was very low in undifferentiated THP-1 cells (not treated with PMA) but up-regulated during differentiation with PMA (Fig. 6). The levels of PPARγ-target gene CD36 and LXRα mRNA were unaffected by HX630 (100 nM) in undifferentiated cells but increased in PMA-differentiated cells (by 1.4- and 1.6-fold, respectively). In parallel with these changes, LXRα-target ABCG1 expression was unaffected by HX630 in undifferentiated THP-1 cells but increased in differentiated cells (by 3.9-fold). PA024 (100 nM) effectively raised ABCG1 and LXRα levels in undifferentiated

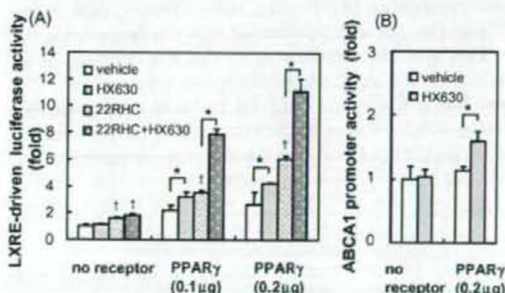

Fig. 5 – HX630 activates LXRE-driven luciferase (A) or ABCA1 promoter (B) gene transcription upon co-expression of PPARγ. RAW264 cells were transfected with an LXREx4-tk-Luc or a pABCA1-Luc reporter plasmid and a phRL-TK internal control in the presence of 0–0.2 μg of PPARγ plasmid and treated for 24 with 100 nM HX630 in the presence or absence of 0.5 μg/ml 22(R)-hydroxycholesterol (22RHC). Luciferase activity in the cell extract was determined and normalized. The values represent the average ± S.D. of three experiments. Significantly different from respective control (*) or vehicle-treated cells (†).

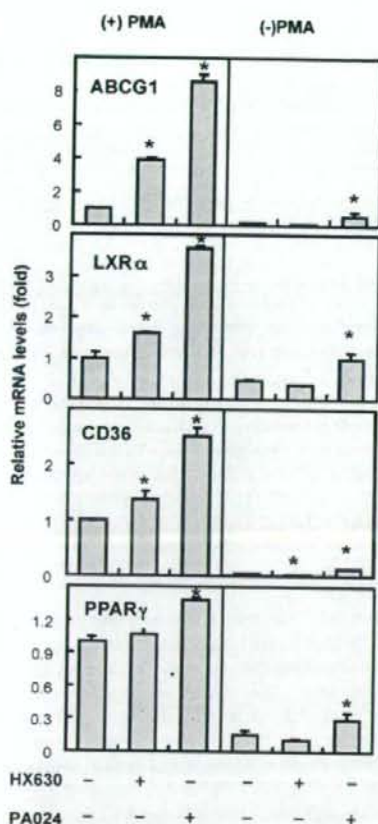

Fig. 6 – PA024 and HX630 increase LXR- and PPARγ-target gene expressions in PMA-differentiated THP-1 cells. PMA-differentiated or undifferentiated THP-1 cells were treated for 24 h with 100 nM PA024 or HX630. The expressions of ABCG1, PPARγ, and LXRα mRNA were measured as described in the legend to Fig. 1B. The values represent the average ± S.D. relative to the PMA-treated control cells (taken as 1) from three experiments. * Significantly different from vehicle-treated cells.

cells, and the effect was drastically increased in PMA-differentiated cells.

4. Discussion

In the present study, we found that RXR agonist PA024 efficiently enhanced ABCA1 mRNA expression in all cell lines tested and strongly promoted apoA-I-mediated cholesterol release (HDL generation) from PMA-differentiated THP-1 cells (Fig. 1B and C). However, HX630 was unable to raise the ABCA1 mRNA level in RAW264 cells (Fig. 1A) and undifferentiated THP-1 cells (Fig. 1B), but was active in differentiated THP-1 cells (Fig. 1B).

The different abilities of the two agonists to induce ABCA1 mRNA expression in RAW264 cells and undifferentiated THP-1 cells were paralleled with their different abilities to activate ABCA1 promoter (Fig. 2). PA024 increased the ABCA1 promoter activity in an LXRE-dependent manner, suggesting that the effect of PA024 was primarily mediated by LXR/RXR activation. Indeed, PA024 strongly stimulated LXRα/RXR-dependent transcription in a cellular transactivation assay (Fig. 3). In contrast, HX630 failed to stimulate the ABCA1 promoter activity. Because HX630 had no capability to directly activate LXR/RXR, suggesting that the failure of ABCA1 induction by HX630 is attributable to this defect.

However, HX630 was able to increase ABCA1 mRNA expression and promote apoA-I-mediated cholesterol release (HDL generation) in PMA-differentiated THP-1 cells (Fig. 1B and C). HX630 was able to induce another LXR-target gene ABCG1 mRNA expression in differentiated cells but not in undifferentiated cells (Fig. 6). These findings suggest that HX630 might increase LXR-target gene transcription in differentiated THP-1 cells.

The LXRα gene is known to be a direct target of PPARγ, and stimulation of PPARγ by agonists has been shown to increase ABCA1 expression by raising LXRα level [30]. We showed that both HX630 and PA024 were able to activate PPARγ/RXR in a cellular transactivation assay (Fig. 4). Furthermore, if PPARγ-expression plasmid was co-transfected into RAW264 cells, HX630 was able to stimulate an LXRE-dependent reporter gene transcription and the ABCA1 promoter activity, as well (Fig. 5). These findings suggest the ability of HX630 to stimulate the PPARγ-LXR-ABCA1 pathway. The level of PPARγ mRNA was greatly induced by differentiation of THP-1 cells with PMA (Fig. 6). In addition, the differentiation augmented HX630-mediated expression of LXR-target ABCA1 and ABCG1 and PPARγ-target LXRα and CD36. Both endogenous PPARγ activity (Fig. 4A and B) and HX630-induced ABCA1 expression (Fig. 1A) were undetectable in RAW264 cells. These findings indicate a close correlation between the ability of HX630 to enhance LXR-target gene expression and the cellular PPARγ mRNA level. The activation of PPARγ/RXR by HX630 primary may elevate LXRα and thereby enhances ABCA1 expression in PMA-differentiated THP-1 cells. Possibility remains that HX630 stimulates LXR/RXR in this cell model. However, we were unable to investigate this possibility due to very low efficiency in DNA transfection in PMA-differentiated cells. Another possibility is that activation of RXR heterodimer(s) other than PPARγ/RXR may also be responsible for the HX630-mediated ABCA1 expression in this cell line. A study has shown that RAR activators increase ABCA1 expression in mouse peripheral macrophages, and that RAR/RXR stimulated the ABCA1 promoter activity via the same DR4 element as LXR/RXR [31]. However, when PMA-differentiated THP-1 cells were treated with RAR agonist AM80 [21], the ABCA1 mRNA level was unchanged (data not shown), suggesting that the RAR/RXR-mediated promoter activation may not make a large contribution to ABCA1 expression in this cell line.

Inductions of ABCA1 and HDL generation by PA024 and HX630 were accompanied by a decrease in the cellular cholesterol level (Fig. 1C). In particular, our findings show the effectiveness of direct activation of LXR/RXR by PA024 (Fig. 3). Although LXR/RXR is known to be permissive in terms

of RXR agonist activation [16,17], HX630 was unable to activate LXR/RXR (Fig. 3). Administration of RXR agonists to mice leads to increases in HDL cholesterol levels [20]. RXR modulators are promising therapeutic strategies [32]. However, increases in serum triglyceride due to RXR agonists are postulated to occur via activation of LXR/RXR, leading to enhanced lipogenesis in response to induction of hepatic SREBP-1c expression [33]. Heterodimer-selective RXR agonists without the ability to directly activate LXR/RXR, may be a promising target for the development of drugs without adverse effects. Synthetic RXR agonists that retain the ability to activate PPARγ/RXR, but have less effect on LXR/RXR and RAR/RXR, have been reported, and pharmacological advantages of these heterodimer-selective RXR agonists as anti-diabetes agents have been demonstrated [34]. The molecular basis of such heterodimer selectivity has not fully been clarified. The mechanism underlying HX630 heterodimer selectivity awaits further investigation.

Acknowledgments

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