which serves as a positive control for GR-mediated up-regulation (Courtois et al., 1999), was also observed in primary hepatocytes. In addition, 10 μM RU-486, which is known to antagonise the effect of dexamethasone on TAT mRNA expression (Runge-Morris et al., 1996), completely repressed the increase in rCES2 mRNA by 100 nM dexamethasone, similar to TAT mRNA (Figure 2). Since 100 nM dexamethasone can activate GR (Runge-Morris et al., 1999), these results suggest that dexamethasone-mediated increase in rCES2 mRNA occurs through GR. Stress-induced glucocorticoid hormones would increase the expression level of rCES2 mRNA in the rat liver, since 10 μM dexamethasone was more effective than 100 nM dexamethasone.

Promoter regions necessary for basal transcription of the *rCES2* gene and for response to dexamethasone were examined on the basis of data from luciferase assays. The results of luciferase assays showed that basal transcriptional activity was almost lost by truncation of the sequence of −73/−7, indicating that the region of −73/−7 is essential for binding of general transcription factors (Figure 5A). In the region of −2957/+51, dexamethasone treatment resulted in activation of the *rCES2* promoter to a level ~2- to 3-fold higher than that of the controls (Figure 5A and 5C), although the effect of dexamethasone on the promoter activity was smaller than that on rCES2 mRNA expression. RU-486 treatment was highly effective in repressing the promoter activation by dexamethasone in the region of −2957/+51 (Figure 5C). Taken together with the results of real-time PCR, these results strongly suggest that GR-mediated transcriptional activation participates in the dexamethasone-mediated increase in rCES2 mRNA in rat hepatocytes. The results of luciferase assays showed that responsivity to dexamethasone was altered in the regions of −73/−7 and−991/−663 (Figure 5B). The response in the region of −73/−7 appeared to be inhibited by RU-486, although the response to dexamethasone was not confirmed by statistical analysis in the experiment using both dexamethasone and RU-486 (Figure 5C). Therefore, nucleotide sequences necessary for dexamethasone-mediated activation of the *rCES2* promoter may be located in the regions of −73/−7 and −991/−663.

It appears that dexamethasone-mediated induction of rCES2 mRNA requires ongoing protein synthesis. When hepatocytes were exposed to 100 nM dexamethasone in the presence of 1 μM cycloheximide, an inhibitor of protein synthesis, the expression level of rCES2 mRNA was repressed by ~50% of that in cells exposed to 100 nM dexamethasone in the absence of cycloheximide, although the difference was not supported by statistical analysis (Figure 3). In contrast, the expression of TAT mRNA was markedly increased by cycloheximide in combination with dexamethasone, in accordance with results of previous studies using cortisol or hydrocortisone acetate (Hofer and Sekeris, 1978; Chesnokov et al., 1990). Although the augmentation of TAT mRNA is not attributed to the inhibition of protein synthesis *per se* (Ernest, 1982), it is conceivable that protein synthesis in rat hepatocytes was somewhat inhibited by 1 μM cycloheximide in the present study, considering that 1 μM cycloheximide treatment for 2 h decreased *de novo* protein synthesis by ~50% in primary rat hepatocytes (Sidhu and Omiecinski, 1998). At 10 μg/mL, cycloheximide completely inhibited dexamethasone-mediated induction of rCES2 mRNA (Figure 4). Cycloheximide at this concentration is known to inhibit *de novo* protein synthesis by more than 90% in human hepatocytes (Gerbal-Chaloin et al., 2002). Therefore these findings suggest that the striking induction of rCES2 mRNA by dexamethasone may require the presence of a protein (s) produced by *de novo* synthesis. A protein(s) necessary for dexamethasone-mediated induction of rCES2 mRNA may be induced in response to dexamethasone if the protein(s) is not short-lived.

In the present study, we demonstrated that dexamethasone enhances transcription of the *rCES2* gene and that GR contributes significantly to dexamethasone-mediated induction of rCES2 mRNA. We also found that another as-yet-unidentified factor(s) may play an essential role in the induction. Thus, we propose that therapeutic compounds that can activate GR induce rCES2.

Declaration of interest

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薬物動態研究におけるヒト多能性幹細胞の活用

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1. はじめに

幹細胞（stem cells）とは，近年よく聞く言葉であるが，自分と同じ細胞を作る能力（自己複製能）と，組織や臓器を構成する多種類の細胞に分化する能力（多分化能）を併せ持った細胞のことである．幹細胞には階層性があり，上位の未分化な幹細胞は自己複製能が高く様々な細胞系列に分化できるが，下位になるに従い自己複製能は失われていき，特定の細胞系列にしか分化できなくなる．胚性幹細胞（embryonic stem cells, ES 細胞）や人工多能性幹細胞（induced pluripotent stem cells, iPS 細胞）は最上位に位置し，生体を構成する全ての細胞に分化する能力を持っていることから多能性幹細胞あるいは万能細胞とも呼ばれている（図 1）．下位には造血幹細胞，間葉系幹細胞，小腸上皮幹細胞，肝幹細胞などの組織幹細胞（体性幹細胞，成体幹細胞とも呼ばれる）がある．組織幹細胞は分化の方向性が決まっているため分化が容易ではあるが，新鮮な組織をヒトから採取し，しかも極わずかしか含まれていない幹細胞を単離しなければならない．一方，多能性幹細胞は，遺伝子操作が可能であり，安定供給にも優れていることから，分化誘導法が確立さえできれば，実験材料としての利用価値は組織幹細胞よりも遥かに大きいことが容易に想像できる．

医薬品の効果や毒性は体内動態に大きく左右されることから，創薬研究において薬物動態試験の重要性はますます高まってきている．従来，薬物動態試験にはラット等の実験動物が多用されてきたが，種差の問題があり，ヒトへの外挿は容易ではない．そこで，より効率よく評価するために，医薬品開発の早い段階からヒトの細胞やオルガネラが薬物動態試験に使用されるようになった．しかし，肝細胞，小腸上皮細胞，腎尿細管上皮細胞等のヒトの試料は，薬物動態試験や毒性試験において有用な実験材料であるが，新鮮な組織や細胞は入手が困難であり，入手できたとしてもロット間差が大きく，数量も限られている．一方，iPS 細胞は目的とする細胞への分

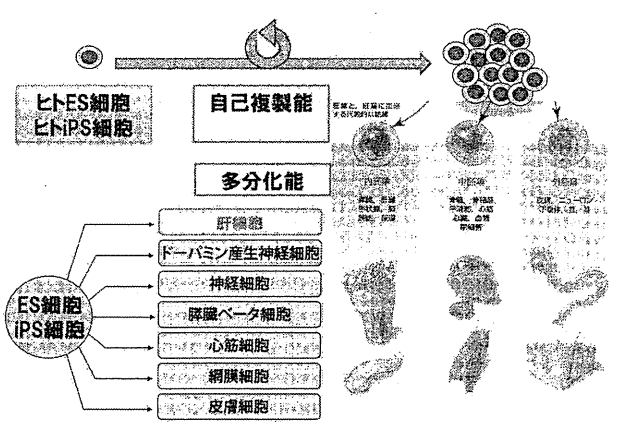

図 1 多能性幹細胞（ES 細胞及び iPS 細胞）の特性

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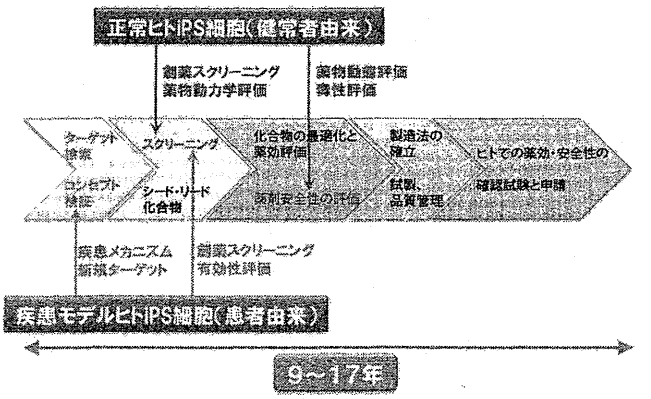

図2　創薬プロセスにおけるヒトiPS細胞の利用

化が確立され，評価系としてのバリデーションが確認できれば，創薬研究の薬物動態評価のみならず新しい実験材料として極めて有用である（図2）．

iPS細胞については，その性質や再生医療等への利用について知る機会が多い半面，規制については誤解されている点も多い．そこで，本稿では，iPS細胞に関する規制と薬物動態研究への応用に向けた取り組みを紹介する．

2. iPS細胞に関する規制

iPS細胞がES細胞と非常に類似した性質を持つことはよく知られている．そのため，法的あるいは倫理的な規制も同じと勘違いされている方が意外と多い．しかし，ES細胞は樹立の際に生命の萌芽である受精卵を壊す（滅失する）のに対し，iPS細胞は線維芽細胞等の体細胞を材料として樹立される点が，法的あるいは倫理的な規制を考える上で根本的に異なる．

ヒトiPS細胞を用いた研究に関わる規制について，①iPS細胞樹立まで，②iPS細胞を用いた研究（非臨床），③iPS細胞を用いた研究（臨床）に分けられるが，本稿では①と②について概略を説明する．

2.1　iPS細胞樹立までに関わる規制

ドナー（患者/健常者）から採取した組織・細胞を用いてiPS細胞を樹立する場合は，被験者の自己決定権，プライバシー及び個人情報の秘密保持，試料の収集・分析・保存/再利用に対する同意，インフォームドコンセント，倫理委員会による審議など「ヘルシンキ宣言」（世界医師会）の人間を対象とする医学研究の倫理的原則に従って行われなければならない．また，ドナー由来のiPS細胞について遺伝子解析を行う場合には，「ヒトゲノム・遺伝子解析研究に関する倫理指針」及び「個人情報の保護に関する法律」を遵守し，解析に当たっては匿名化が原則となる．その際には，連結可能匿名化（結果と提供者を結び付けることが可能）と連結不可能匿名化（結果と提供者を結び付けることが不可能）があり，どちらも倫理委員会の承認が必要である．

一方，ヒト皮膚線維芽細胞等iPS細胞の樹立に用いる細胞を表1の様な公的機関や会社等から入手することも可能である．ただし，このような細胞を使用する際には，契約書を交わす場合があり，また細胞によっては寄託者の承認や使用目的に制限がある場合もある．

近年は，iPS細胞樹立にウイルスを用いない方法も開発されているが，最も一般的なレトロウイルスを用いた場合を例に挙げると，以下の規制が関わってくる．すなわち，大腸菌によるプラスミドの増幅（ウイルス/非ウイルス）とウイルスベクター感染によるiPS細胞誘導がある．そのために，「遺伝子組換え生物等の使用等の規制による生物多様性の確保に関する法律」（カルタヘナ法）（研究での使用は「二種使用」）と「研究開発等に係る遺伝子組換え生物等の第二種使用等に当たって執るべき拡散防止措置等を定める省令」及び「研究開発段階における遺伝子組換え生物等の第二種使用等の手引」が関与することになる．なお，レトロウイルスベクター（増殖力等欠損株）感染によるiPS細胞誘導時には，P2レベルの核酸防止措置が必須となるが，樹立後は通常P2レベルで行う必要はない．

2.2　樹立したヒトiPS細胞を用いた非臨床研究に関わる規制

樹立されたiPS細胞を非臨床研究に用いる場合，生殖細胞への分化誘導と特定胚（クローン胚を含む）

表1　実験用細胞の入手先

Cell applications Inc.	(http://www.cellapplications.com/)
Lonza	(http://www.lonza.com/group/en.html)
American Type Culture Collection	(ATCC：http://www.atcc.org/)
理研バイオリソースセンター	(http://www.brc.riken.jp/)
医薬基盤研究所	(JCRB：http://www.nibio.go.jp/index.shtml)
ヒューマンサイエンス研究資源バンク	(HSRRB：http://www.jhsf.or.jp/bank/cell.html)

の作成に関する研究を除き，その扱いはHepG2細胞やHEK293細胞等，通常研究で用いられている培養細胞と同じである．この点は，「ヒトES細胞の使用に関する指針」に規定されている様に，例え使用研究であっても機関内又は他の使用機関の倫理審査委員会の審査を受けた後に使用計画の届け出を文部科学大臣に出さなければならないES細胞研究とは大きく異なる．

生殖細胞への分化誘導には，「ヒトiPS細胞又はヒト組織幹細胞からの生殖細胞の作成を行う研究に関する指針」，「ヒトiPS細胞又はヒト組織幹細胞からの生殖細胞作成における研究計画の実施の手引き」及び「ヒトiPS細胞又はヒト組織幹細胞からの生殖細胞の作成を行う研究に関する指針」があり，作成した生殖細胞でヒト胚を作成しないことや大臣への届け出等が義務付けられている．また，特定胚の作成に関する規制については，「ヒトに関するクローン技術等の規制に関する法律」(クローン規制法)，「ヒトに関するクローン技術等の規制に関する法律施行規則」及び「特定胚の取扱いに関する指針」がある．

特定胚のうち，現時点で作成が可能なのは，人クローン胚と動物性集合胚であるが，どちらも特定胚を作成することについての提供者による同意が必要である．また，これら研究の胚の取扱期間は，原始線条が現れるまで，または14日以内のどちらか短い方となっている．したがって，ヒトの特定胚を使った薬物動態試験などは余程発生初期段階での解析でなければ現段階では不可能である．

3. 薬物動態試験への応用と期待

ヒトiPS細胞の薬物動態試験への応用という観点から，薬物動態において重要な細胞である肝細胞様細胞および小腸上皮細胞様細胞への分化誘導に関して，最近の報告に著者らの結果を踏まえて紹介したい．

3.1　ヒトiPS細胞の肝細胞様細胞への分化誘導法

ヒトiPS細胞の肝細胞様細胞への分化誘導研究は，先行していたES細胞の分化誘導法を参考として，胚体内胚葉への分化，肝芽細胞様細胞への分化，肝細胞様細胞への分化・成熟の3つの段階に大きく分けて，複数の因子を段階的に添加していくことで行われている．ヒトES細胞から肝細胞様細胞への分化誘導は，2003年にRambhatlaらによって初めて報告された[1]．その後，より効率的に分化させるために改良された方法が現在までに多数報告されている[2,3]．ヒトiPS細胞の肝細胞様細胞への分化については，2009年のSongら[4]の報告が最初であり，それに引き続いていくつか報告されている[5]．

動物の体を構成する臓器が発生過程でどのようなメカニズムで統一された形づくりをするのかをツメガエルやイモリ卵を使って*in vitro*で研究されてきた．胞胚期のアニマルキャップ（未分化細胞塊）にtransforming growth factor-β（TGF-β）superfamilyのひとつであるactivin Aを処理すると濃度依存的に様々な器官や組織を分化誘導する．低濃度では血球や体腔内上皮，中濃度は筋肉，高濃度では脊索を分化誘導する．さらに高濃度では心臓や小腸，肝臓といった内胚葉性器官も分化誘導する．また，ヒトES細胞の場合でも同様に高濃度のactivin A(100 ng/mL）を処理することで効率的に胚体内胚葉へ誘導される．このような知見から，ヒトES細胞やiPS細胞の胚体内胚葉への分化のほとんどにactivin Aが用いられる．このとき，Wnt familyのひとつであるWnt3aは内胚葉や中胚葉の分化に重要であり，効率よくかつ速やかに肝細胞様細胞への分化が進むとして，activin Aと併用される場合もある．しかし，著者らはWnt3aが特に分化を促進するとの結果を得ることは出来なかったことから，通常activin Aのみを使用している．

肝臓の初期発生において，腹側前腸内胚葉が近接する心臓中胚葉からのfibroblast growth factor(FGF)

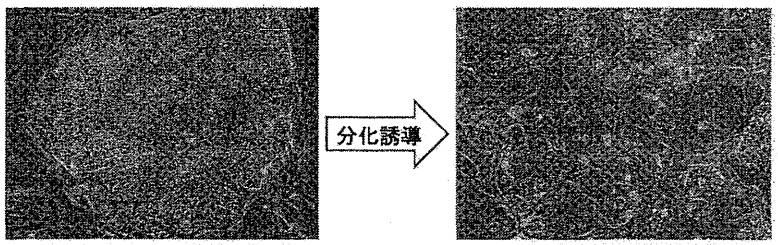

図3　ヒトiPS細胞とiPS細胞由来肝細胞様細胞の顕微鏡写真

シグナルや横中隔からのbone morphogenetic protein（BMP）シグナルによって肝芽細胞への分化が誘導される．そのため，胚体内胚葉から肝芽細胞様細胞への分化の過程にFGFやBMPがよく用いられる．一方，興味深いことに溶媒として多用されるdimethyl sulfoxide（DMSO）も肝芽細胞様細胞や肝細胞様細胞へ分化させることが知られている[2]．

この段階は研究者により様々であるが，多くは上記因子の複数あるfamilyを色々と組み合わせて行われている．著者らもこれら液性因子を組み合わせて分化を試みたが，分化効率にあまり差がなく，DMSOのみを使用した場合とも大きな差は認められなかった．また，DMSOは液性因子よりはるかに安価で取扱いも容易なことから，今では専らDMSOを使用している．この方法で分化した場合でも，ヒトES細胞での結果であるが成人様の薬物応答性を示す肝細胞様細胞を得ることができた[6]．

Hepatocyte growth factor（HGF）は，肝再生において成熟肝細胞の増殖因子として発見されたものであるが，肝臓形成中間期（mid-stage hepatogenesis）において胎児肝細胞の増殖・維持に関係している．また，マウス胎仔初代肝細胞をinterleukin-6（IL-6）familyのサイトカインであるoncostatin M（OSM）とdexamethasone（DEX）を含む培地で培養すると未熟な肝細胞を成熟させることが示された．このようなことから，最終の成熟段階において多くの研究者はHGF, OSM, DEXを添加している．しかし，成熟と言ってもこの組み合わせで胎児様の肝細胞には誘導されるが，それ以降の成熟は難しい．近年，著者らは様々な分化誘導法の工夫を行い，肝細胞マーカーのalbumin（ALB）のmRNA発現レベルは，成人肝培養細胞と同程度の発現が認められるまでになっている．しかし，胎児肝に特異的に発現するα-fetoprotein（AFP）やCYP3A7も高発現しているため，現段階では胎児肝レベルであり十分に成熟しているとは言えない．これは著者らの研究に限らず肝細胞への分化に関する報告はどれも成熟化が問題となっており，今後薬物動態試験に使用するために克服しなければならない必須の課題である．

液性因子だけでは限界があるとして近年新たな試みがなされている．それは肝発生において重要な転写因子のひとつであるhematopoietically expressed homeobox（HEX）[7]や肝の機能獲得に重要なhepatocyte nuclear factor 4α（HNF-4α）をヒトiPS細胞の分化のある時期に各々一過性に過剰発現する方法であり，従来の方法に比べ，短期間かつ高効率に肝細胞様細胞を作成している．この場合も，胎児様の性質を残していることが課題として挙げられているが，肝発生において重要な役割を果たしている遺伝子の導入は，効率的な肝細胞様細胞の新たな分化誘導手段として今後利用されることが予想される．

3.2　ヒトiPS細胞由来肝細胞様細胞の特徴

形態学的な特徴として，未分化なiPS細胞は細胞質がほとんどないため核の占める割合が大きく，核小体が明瞭である．また，ヒトiPS細胞は単層から成るコロニーを形成し，そのコロニーの境界線は滑らかで明確である．ヒトiPS細胞にactivin Aを処理すると非常に多くの細胞が死ぬが，残った細胞は大きくなり，縁が尖った形態を示す．分化に伴い更に大きくなり，最終段階では肝細胞に特徴的な多核の細胞が出現する（図3）．

遺伝子の発現は，activin Aの処理により未分化マーカーの発現が低下し，内胚葉のマーカーであるforkhead box A2（FOXA2），sex determining region Y box 17（SOX17）の発現が誘導される．さらに分化が進むとAFP, ALB, tyrosine aminotransferase（TAT）などの肝細胞マーカーに加えて，薬物代謝の主要酵素であるCYP3A4の発現量が増加する．ま

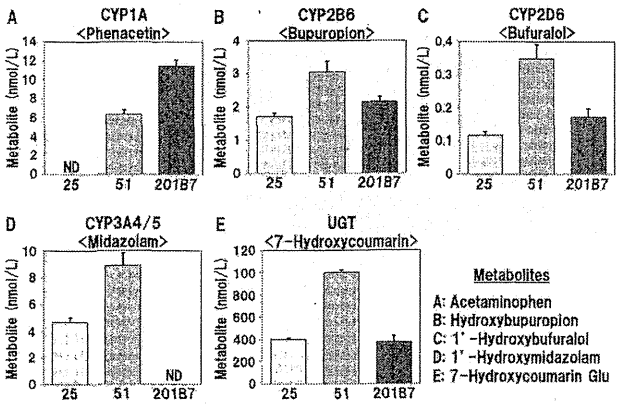

図4　ヒトiPS細胞から分化させた肝細胞様細胞の薬物代謝活性

Mean＋S.D.(n＝3)，25：ヒトiPS細胞#25株，51：ヒトiPS細胞#51株，201B7：ヒトiPS細胞201B7株

た，肝細胞に特異的な機能であるALBの分泌やインドシアニングリーンの取り込み等も認められる．

3.3　ヒトiPS細胞由来肝細胞様細胞の薬物代謝活性

ヒトiPS細胞から誘導した肝細胞様細胞におけるCYPの発現やその代謝活性は成人肝培養細胞と比較して顕著に低いがmethylcholanthrene（CYP1Aの誘導剤），phenobarbital（CYP2Bの誘導剤），rifampicin（CYP3A4の誘導剤）により誘導され，各々代謝活性の上昇がみられる[1,4,7]．その程度はCYP分子種によっても異なるが，2～5倍程度である．Bufuralolを基質としてヒトiPS細胞から分化誘導した肝細胞様細胞の代謝特性について検討を行った報告では，初代培養肝細胞と同様の代謝物が生成され，その代謝物はCYPによるものだけでなく，UDP-glucuronosyltransferase（UGT）やglutathione *S*-transferase（GST）などphase IIの代謝酵素によって生成されるグルクロン酸抱合体やグルタチオン抱合体なども検出されている[3]．また，pregnane X receptor（PXR），constitutive androstane receptor（CAR），aryl hydrocarbon receptor（AhR），liver X receptor α（LXRα）などの核内受容体に加え，multidrug resistance protein 1（MDR1），organic anion transporting polypeptide 2（OATP2）等の薬物トランスポーターの発現もみられていることから，薬物動態関連の機能をある程度獲得していると考えられる．

著者らはヒトiPS細胞の肝細胞様細胞への分化誘導を第一段階としてactivin Aで内胚葉，肝芽細胞への分化にDMSOを用い，成熟段階をHGF，OSM，DEX，インスリンで行っている．図4はヒトiPS細胞3株を同じ条件で同時に分化させた細胞における薬物代謝活性を比較したものである[8]．株によって差はあるものの，CYP1A，CYP2B6，CYP2D6，CYP3A4およびUGTの活性が検出された．さらに，結果は示していないがsulfotransferase（SLT）活性も検出されており，薬物動態試験への利用の可能性が示唆された．

3.4　ヒトiPS細胞から小腸上皮細胞様細胞への分化

現在使用されている医薬品の大部分は経口薬であり，経口投与された医薬品が全身循環に移行するためには小腸粘膜を通過する必要がある．小腸上皮には薬物トランスポーターや薬物代謝酵素が存在し，これらが医薬品のバイオアベイラビリティーに影響を及ぼすことから，小腸は肝臓と同様に薬物動態に影響する主要な臓器となっている．したがって，小腸における医薬品の吸収・排泄や代謝を解析・評価することは非常に重要である．現在，肝臓については，ヒト凍結肝細胞や初代肝細胞など組織由来の細胞が利用可能であるが，医薬品候補化合物の吸収・排泄特性を調べるためにイヌ腎由来MDCK(Madin-Darby canine kidney）細胞株やヒト結腸ガン由来Caco-2細胞単層膜による薬物透過試験が行われている．また，腸管は医薬品の吸収部位でもあるが，CYP3A4やUGTなど薬物代謝酵素等も多く発現しており，グレープフルーツジュース等による阻害やリファンピシン等による誘導等の食品や医薬品との相互作用が問題となる点でも重要である．しかし，MDCK細胞やCaco-2細胞は小腸上皮細胞とは本来異なる性質を持つ細胞であるために，適正な評価と予測が困難である．

肝細胞と同様にヒトiPS細胞から小腸上皮細胞へ分化誘導し，薬物動態の評価系としての利用が可能となれば有用なツールとなるが，腸管への分化に関する報告は肝細胞への分化の報告に比べて極めて少ない．2009年にマウスES細胞での方法を応用し，初めてマウスiPS細胞から三胚葉よりなる胚様体を形成して腸管組織への分化誘導に成功している．また，ヒトiPS細胞を用いた分化誘導に関しても最近報告されている[9]．しかし，これらは形態学的に腸管組織としての特徴を有するが，薬物動態学的な解

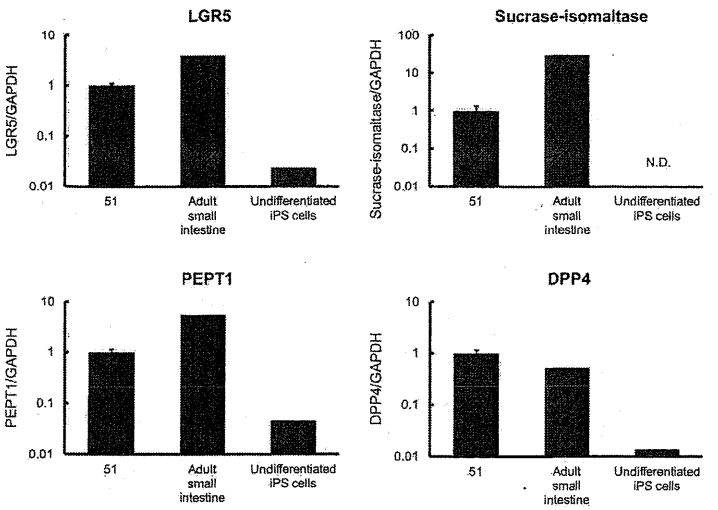

図5　ヒトiPS細胞から分化させた腸管上皮細胞様細胞のmRNA発現量
Mean＋S.D.(n＝3), 51：ヒトiPS細胞 #51株由来腸管上皮細胞様細胞, N.D.: not detected. LGR5: leucine-rich repeat containing G protein-coupled receptor 5, PEPT1: oligopeptide transporter 1, DPP4: dipeptidyl-peptidase 4.

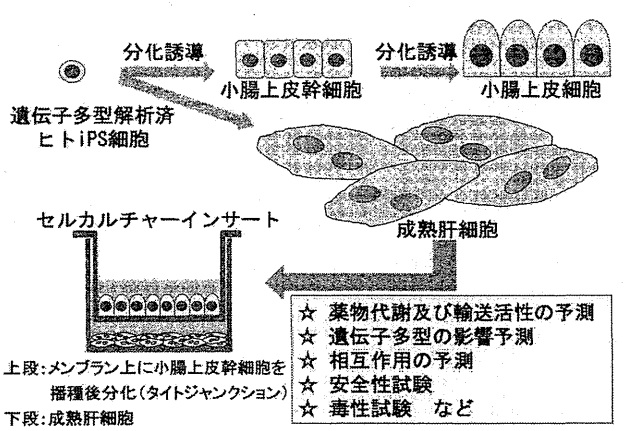

図6　ヒトiPS細胞から小腸上皮細胞及び肝細胞の分化誘導と薬物動態試験

析に応用できるまでの機能評価はされておらず，ほとんど研究が進んでいないのが現状である．

著者らもヒトiPS細胞から内胚葉を経由した腸管上皮細胞様細胞への分化について検討を行っている．その結果，腸管幹細胞に局在するとされるleucine-rich repeat containing G protein-coupled receptor 5（LGR5），腸管上皮細胞に特異的に存在するsucrase-isomaltaseやdipeptidyl-peptidase 4（DPP4）の発現に加え，ペプチドトランスポーターであるoligopeptide transporter 1（PEPT1）の発現も確認されたことから，腸管組織細胞への分化が確認された（図5）[8]．今後は，より効率的な分化法の検討に加え，薬物代謝能や輸送能に関する機能解析を行っていく予定である．

4. さ い ご に

多くの場合医薬品は経口投与されることから，薬物の吸収及び代謝に関与する主要な臓器である小腸や肝臓での薬物動態をより正確かつ簡便に評価することは，薬物の有効性や安全性の予測に極めて重要である．したがって，小腸から肝臓までの薬物吸収・代謝の評価を一体化して評価できる実験モデル系の構築が望まれる．しかし，小腸上皮細胞と肝細胞を用いて，小腸と肝臓を結ぶモデル系を構築することは，これまでヒト小腸上皮細胞が容易に入手できないために困難であった．しかし，肝細胞同様ヒトiPS細胞から小腸上皮細胞を分化誘導にて作成することが出来ればセルカルチャーインサートを用いた一体型のモデル系も可能と考える（図6），さらに，将来的にはチップ上にiPS細胞由来の小腸上皮細胞と肝細胞をつなぐ微小な流路や反応室，混合室を設け，細胞間相互作用を解析する細胞機能解析チップが出来れば，生化学分析デバイスとして，基礎研究や創薬分野において，有用なツールとなると期待される．小腸上皮細胞への分化は世界的にも始まったばかりではあるが，これが実現できればより優れた動態特性を有する安全で有用な医薬品の開発にも貢献できるものと考えられる．また，薬物代謝酵素や薬物トランスポーターの遺伝子多型が薬物動態に影響し，

薬効や副作用発現の個人差の原因の１つとなっていることが明らかになっている．これまでの評価系では遺伝子多型の評価は困難であったが，遺伝子多型が明らかな体細胞からiPS細胞を樹立すれば，遺伝子多型の薬物動態に及ぼす影響の予測も容易になるものと考えられる．著者らはこのようなことを夢見て，肝細胞と小腸上皮細胞への分化について研究を行っている．

著者らが使用したヒトiPS細胞は，国立成育医療研究センターおよび京都大学より供与していただきました．また，研究を遂行するにあたりご協力いただいた国立医薬品食品衛生研究所　医薬安全科学部　黒瀬光一室長，田辺三菱製薬株式会社　研究本部　薬物動態研究所　丹羽卓朗氏に感謝致します．

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Note

Mechanisms of CYP3A Induction by Glucocorticoids in Human Fetal Liver Cells

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Summary: Human fetal liver (HFL) cells express major drug metabolic enzymes CYP3A4, CYP3A5 and CYP3A7. In the fetal hepatocytes, betamethasone and dexamethasone (DEX) markedly enhanced the expression levels of CYP3A4 and CYP3A7 mRNAs and slightly increased the expression level of CYP3A5 mRNA. Interestingly, a high correlation between the CYP3A induction ability and the intensity of anti-inflammatory effect was observed. Human glucocorticoid receptor (GR)–small interfering RNA clearly attenuated the expression level of GR mRNA, and diminished the DEX-stimulated CYP3A4, CYP3A5 and CYP3A7 expression in HFL cells. These findings indicate that GR mediates the induction of CYP3A4 and CYP3A7 expression in human fetal hepatocytes as well as the CYP3A5.

Keywords: CYP3A; induction; glucocorticoid; human fetal liver cells; glucocorticoid receptor; small interfering RNA; specific small interfering RNA

Introduction

Cytochrome P450 (CYP) comprises a gene superfamily of hemoproteins that catalyze the oxidation of lipophilic substrates to more water-soluble products. One of them, the human CYP3A subfamily, contains mainly four isoforms, CYP3A4, CYP3A5, CYP3A7, and CYP3A43. In particular, CYP3A4, CYP3A5 and CYP3A7, highly expressed in liver, associate with the metabolism of many compounds. The expression levels of CYP3A isoforms are enhanced by treatment with various agents, such as rifampicin (RIF), phenobarbital, clotrimazole, and dexamethasone (DEX).[1–3] We previously clarified that CYP3A4 and CYP3A7 mRNA expression levels were markedly up-regulated by DEX, but not by RIF, in human fetal liver (HFL) cells.[4] These data suggested that the mechanisms of CYP3A induction in HFL differed from those in adult liver.

The glucocorticoid-induced CYP3A5 expression is mediated by glucocorticoid receptor (GR) signaling.[5,6] As previously described, transcriptional activation of the *CYP3A4* gene by glucocorticoids is also known to occur through two distinct mechanisms involving GR: first by controlling the expression of pregnane X receptor (PXR) under physiological conditions through the classical GR pathway, and second by activating PXR under bolus or stress conditions.[7,8] The role of GR in CYP3A4 regulation is, however, unclear, and an accurate assessment of whether GR plays a direct and/or indirect role remains obscure.[9] We have reported that concomitant treatment with RU486, a GR antagonist, suppressed DEX-mediated induction of CYP3A4, CYP3A5, and CYP3A7 expression completely in HFL cells.[4] These data suggested that GR was required in the CYP3A4 induction.

Introduction of specific small interfering RNA (siRNA) in cells has been shown to specifically knock down the target

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gene expression.[10] Recently, Matsubara *et al.*[11] reported that adenovirus vector expressing human PXR-siRNA (AdhPXR-siRNA) was a potent tool to discern the role of PXR in the chemical-mediated activation of the *CYP3A4* gene. The system could be useful for assessment of a variety of nuclear receptor functions *in vivo* and *in vitro*.

In the present study, to further characterize whether GR is involved in DEX-mediated activation of *CYP3A4* and *CYP3A7* genes as well as the *CYP3A5* gene, we investigated the effect of glucocorticoids on the CYP3A expression and the role of GR in the induction of the *CYP3A* gene expression in HFL cells using the AdhGR-siRNA system.

Materials and Methods

Materials: SuperScript II first-strand synthesis system for reverse transcription–polymerase chain reaction (RT-PCR) and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA, USA); DEX, hydrocortisone, prednisolone and betamethasone were obtained from Wako Pure Chemicals (Osaka, Japan); fludrocortisone and methylprednisolone were obtained from MP Biomedicals (Costa Mesa, CA, USA); cortisone and Williams' medium E were obtained from Sigma Chemical Co. (St. Louis, MO, USA); *TaKaRa* EX Taq was obtained from Takara-Bio (Otsu, Japan); and PCR primers were purchased from Sigma Genosys (Hokkaido, Japan). All other reagents used were of the highest quality available.

Human fetal liver cells: HFL cells were obtained from Applied Cell Biology Research Institute (Kirkland, WA, USA). The cell culture was initiated from a pool of six normal human liver tissues (average gestation 13 weeks) by elutriation following dispase digestion of tissue. The cell culture was cryopreserved at −150°C until use after proliferation.

Cell culture and drug treatment: HFL cells were incubated on dishes coated with type I collagen. Williams' medium E (Sigma Chemical Co.) containing 10% (v/v) fetal bovine serum, antibiotics (50 μg/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL neomycin), and 2 mM L-glutamine was used for culture of HFL cells under the condition of 5% CO_2 at 37°C. The medium was exchanged every 24 h.

Induction of CYP3A mRNA by representative glucocorticoids: HFL cells were cultured for 7 days after seeding of 5,000 cells/well onto 6-well culture plates, and then treated with 10 nM cortisone, hydrocortisone, prednisolone, methylprednisolone, fludrocortisone, betamethasone or DEX for 72 h. The compounds were dissolved in dimethyl sulfoxide (DMSO), which was added to the culture medium at a final concentration of 0.1%. The medium was replaced daily with fresh medium containing either test compounds dissolved in vehicle or vehicle alone. After treatment, total RNA was prepared from the cells.

Construction of siRNA-expressing adenovirus: Human H1 RNA gene promoter was used for the expression of siRNA. AdhGR-siRNA was constructed with AdEasy™ System (MP Biomedicals, Irvine, CA, USA) according to the manufacturer's protocol. The hGR-specific siRNA, designed by Takara-Bio, was amplified by PCR with primers 5′-CGCGTCGACATGACCCTACTGCAGTACTTTCAAGAGAAG-3′ and 5′-CGCAAGCTTAAAAAAATGACCCTACTGCAGTACTTCTCTTGAAAG-3′. PCR product was digested with *Sal* I and *Hind* III, and ligated into the same restriction sites of pShattle-H1. AdCont (AxCALacZ), which expresses β-galactosidase, was provided by Dr. Izumi Saito (Tokyo University). The titer of adenovirus, 50% titer culture infections dose ($TCID_{50}$), was determined in HEK293 cells. The value of $TCID_{50}$ was reported to be almost equivalent to that of plaque-forming units.[12] Multiplicity of infection (MOI) was calculated by dividing $TCID_{50}$ by the number of cells.

Effects of GR knockdown by adenoviral hGR-siRNA expression on CYP3A induction by DEX: HFL cells were cultured for 1 day after seeding 100,000 cells/well onto 6-well culture plates, and then were infected with AdhGR-siRNA (MOI of 10 or 50). The HFL cells cultured for 3 days after infection were incubated with Williams' medium E containing vehicle (0.1% DMSO) or 100 nM DEX for 2 days. We used AdCont as a control for RNA knockdown experiments. Thereafter, total RNA was prepared from HFL cells and CYP3A mRNA was analyzed by semiquantitative RT-PCR.

RNA extraction and semiquantitative RT-PCR analysis: Total RNA was extracted from the treated cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was generated from 2 μg total RNA. Reverse-transcription reaction was performed using a SuperScript II (Invitrogen) according to the manufacturer's instructions. With the cDNA obtained, PCR was carried out using a MyCycler thermal cycler (Bio-Rad, Hercules, CA, USA) and PCR Express thermal cycler (Hybaid, Middlesex, UK). The primers and amplification conditions used are summarized in **Table 1**. The amplified products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. The levels of these mRNAs were quantified from their band densities on the agarose gels using Printgraph AE-6914 and Scion Image Software (by Dr. W. Rasband, http://www.scioncorp.com/), and were normalized relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Results and Discussion

Effects of representative glucocorticoids on expression of CYP3A mRNA: Anti-inflammatory effects of glucocorticoids are caused by binding of GR homodimers to glucocorticoid response elements in the promoter region of steroid-sensitive genes, which may encode anti-inflammatory proteins.[13] If GR mediates the CYP3A induction, the glucocorticoid-mediated change in CYP3A expression could be highly correlated with the anti-inflam-

Table 1. PCR primers and conditions

Primer	Length/ annealing temperature	Sense primer 5′→3′	Antisense primer 5′→3′
CYP3A4	626 bp/60°C	CTGTGTGTTTCCAAGAGAAGTTAC	ACCTCATGCCAATGCAGTTT
CYP3A5	239 bp/62°C	TGACCCAAAGTACTGGACAG	TGAAGAAGTCCTTGCGTGTC
CYP3A7	475 bp/54°C	CTATGATACTGTGCTACAGT	TCAGGCTCCACTTACGGTCT
GR	557 bp/54°C	ACACAGGCTTCAGGTATCTT	ACTGCTTCTGTTGCCAAG
PXR	442 bp/68°C	CAAGCGGAAGAAAAGTGAACG	CTGGTCCTCGATGGGCAAGTC
GAPDH	307 bp/54°C	CATCACCATCTTCCAGGAGC	CATGAGTCCTTCCACGATACC

Table 2. Relative anti-inflammatory potencies of representative glucocorticoids and their ability to induce CYP3As

Compound	Anti-inflammatory potency[a]	Induction ability (CYP3As/GAPDH)[b]		
		CYP3A4	CYP3A5	CYP3A7
Control (DMSO)	—	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.02
Cortisone	0.8	0.67 ± 0.05	0.63 ± 0.05	0.91 ± 0.26
Hydrocortisone	1	0.71 ± 0.05	0.62 ± 0.02	1.10 ± 0.18
Prednisolone	4	1.38 ± 0.23	0.93 ± 0.03	2.41 ± 0.65
Methylprednisolone	5	1.53 ± 0.12	0.49 ± 0.05	1.73 ± 0.54
Fludrocortisone	10	1.82 ± 0.30	0.64 ± 0.08	2.43 ± 0.57
Betamethasone	25	2.90 ± 0.51	1.75 ± 0.16	4.33 ± 0.80
Dexamethasone	25	3.75 ± 1.27	1.87 ± 0.09	3.79 ± 1.60

[a]Relative to hydrocortisone, which is assigned a value of 1.[13]
[b]HFL cells were cultured for 7 days after seeding 5,000 cells/well onto 6-well culture plates, and then treated with 10 nM glucocorticoids for 72 h. After treatment, total RNA was prepared from the cells. The mRNA levels were calculated using semiquantitative RT-PCR analysis as described in Materials and Methods. The values are expressed as the mean ± standard deviation of three experiments.

matory effect of the glucocorticoids. Thus we investigated whether representative glucocorticoids used in clinical medicine induce CYP3A4, CYP3A5 and CYP3A7 expression in HFL cells (**Table 2**). The levels of CYP3A4 and CYP3A7 mRNAs were markedly enhanced by treatment with 10 nM of betamethasone or DEX, which are classified as the most potent glucocorticoids. The expression level of CYP3A5 mRNA was also enhanced, but the induction of CYP3A5 expression was slight, compared to that of the CYP3A4 and CYP3A7. Generally, a good correlation has been found between the affinity for the cytosolic GR and anti-inflammatory potencies of glucocorticoids.[14] Interestingly, a high correlation between the inducibility and the anti-inflammatory potencies of glucocorticoids[15] was observed, especially for CYP3A4 ($r^2 = 0.92$) and CYP3A7 ($r^2 = 0.91$) (**Fig. 1**). These results may indicate that GR mediates the induction of CYP3As in HFL cells. Glucocorticoids might induce the expression of CYP3As by complexing with GR, undergoing nuclear translocation, interacting with DNA *cis* sequences and modulating gene transcription.

Effects of adenoviral hGR-siRNA expression on CYP3A induction by DEX: Expression levels of CYP3A4, CYP3A5 and CYP3A7 mRNAs were increased 6.9-, 1.6- and 5.3-fold, respectively, of the control by treatment with 100 nM of DEX (**Figs. 2A** and **2C**).

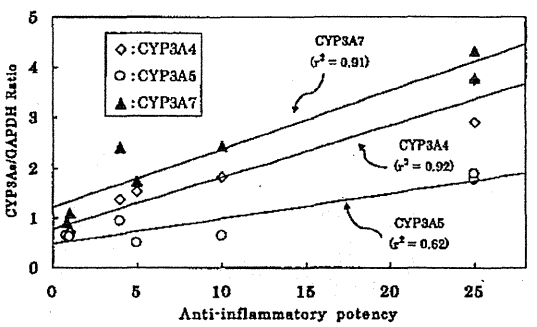

Fig. 1. Correlation between the anti-inflammatory potencies of glucocorticoids and the CYP3A-induction ability

The molecular mechanism of induction is best understood for CYP3A4. The first 1 kb of the 5′-flanking regions of CYP3A4 and CYP3A7 share 91% sequence similarity.[16] However, the 5′-flanking region of CYP3A5 (−1 to −1,434 bp) shares 60 and 59% sequence similarity to that of CYP3A4 and CYP3A7, respectively.[17] The low homology might be one of the factors of weak induction of CYP3A5.

To discern the involvement of GR in the induction process of CYP3As by DEX, AdhGR-siRNA was introduced to specifically knock down the target gene expression in the cells (**Fig. 2**). GR mRNA was detected in HFL cells and the expression was suppressed to less than 50% of that in the control by the expression of hGR-siRNA (**Figs. 2A** and **2B**). Introduction of AdhGR-siRNA almost completely inhibited the DEX-mediated induction of CYP3A4 and CYP3A5 mRNAs (**Fig. 2C**). On the other hand, the induction of CYP3A7 mRNA was suppressed to about 60% of AdCont used as a control for RNA knockdown experiments. We do not know the reason why the induction of CYP3A7 mRNA was not completely suppressed by hGR-siRNA (**Fig. 2C**). This phenomenon might be caused by the different structure of the 5′-flanking regions of CYP3A4 and CYP3A7, including GR responsive elements.

Chemical-induced expression of the *CYP3A4* gene can be mediated by PXR heterodimerized with retinoid X receptor through binding to the *CYP3A4* 5′-flanking region.[18–20] In the present study, both 10 and 100 nM of DEX showed clear induction of CYP3A4 and CYP3A7 expression in HFL cells (**Table 2** and **Fig. 2C**), concentrations sufficient to activate GR but not PXR.[7] We have reported that PXR mRNA is

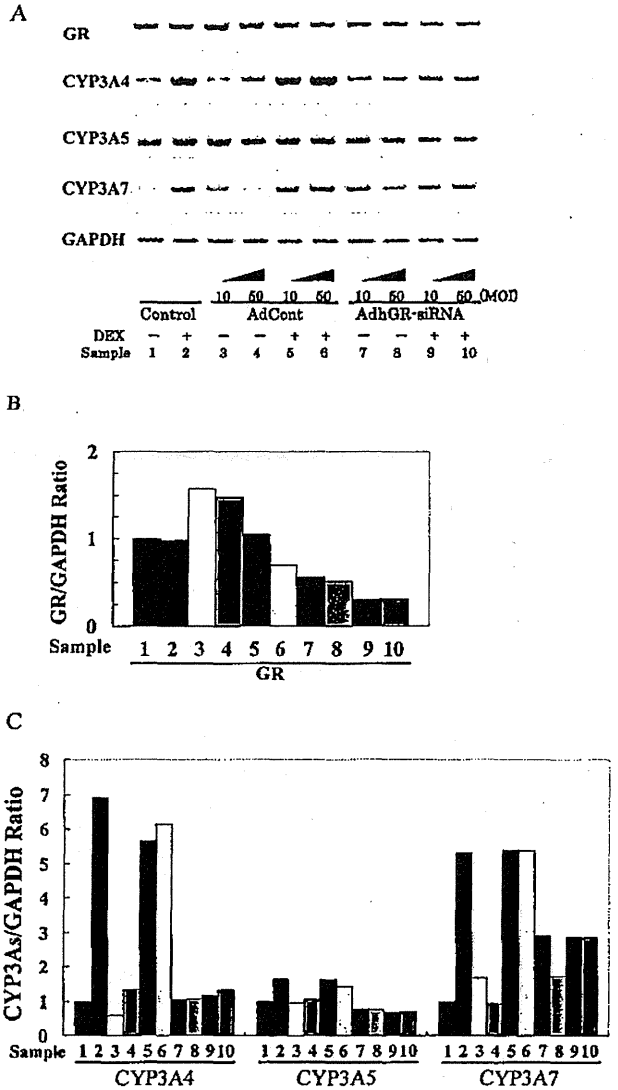

Fig. 2. (Color online) Effects of adenoviral GR-siRNA expression on GR, CYP3A4, CYP3A5 and CYP3A7 mRNA levels

HFL cells were cultured for 1 day after seeding 100,000 cells/well onto 6-well culture plates, and then were infected with AdhGR-siRNA (MOI of 10 or 50). We used AdCont as a control for RNA knockdown experiments. The HFL cells cultured for 3 days after infection were incubated with Williams' medium E containing vehicle (0.1% DMSO) or 100 nM DEX for 2 days. Then, total RNA was prepared from HFL cells and GR, CYP3A4, CYP3A5 and CYP3A7 mRNAs were analyzed by semiquantitative RT-PCR as described in Materials and Methods. A: An image of ethidium bromide-stained agarose gel is shown. Data presented are the ratio of B: GR, and C: CYP3A4, CYP3A5, and CYP3A7 mRNA to GAPDH normalized at 1.0 for DMSO treatment alone in the absence of adenovirus (Sample 1). The columns present the mean of two individual experiments.

not detected by RT-PCR in HFL cells.[21] Furthermore, insufficient RIF-mediated CYP3A4 induction was observed in HFL cells with only PXR overexpression, maybe because of lower expression of some crucial transcription factors such as hepatocyte nuclear factor 4α (HNF4α) and peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) in the HFL cells.[22] More recently, Pang *et al.* reported that glucocorticoids, including dexamethasone, cortisol, corticosterone, and cortisone, all induced the expression of CYP3A7 mRNA, whereas RIF had no effect on CYP3A7 expression in double-transgenic mice expressing human PXR and CYP3A4/7. They suggested that CYP3A7 is developmentally regulated in mouse liver primarily by glucocorticoids through the GR.[23] These results strongly support a view that DEX induces CYP3A expression through GR, but not PXR, in human fetal liver.

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Regular Article

Effects of Hypoxia-inducible Factor-1α Chemical Stabilizer, $CoCl_2$ and Hypoxia on Gene Expression of CYP3As in Human Fetal Liver Cells

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Summary: Distinctive response patterns of CYP3A4 and CYP3A7 to cobalt chloride ($CoCl_2$) in human fetal liver (HFL) cells were observed and compared with those under hypoxic conditions. The expression levels of CYP3A4 and CYP3A7 mRNAs were decreased by $CoCl_2$ and hypoxia, although significance could not be determined in HFL cells cultured under 3% O_2. The hypoxia-inducible factor-1α (HIF-1α) protein content in HFL cells was significantly increased by $CoCl_2$ and 3% O_2. Transcriptional activities of CYP3A4 and CYP3A7 were not altered by 3% O_2 when reporter plasmids containing the promoter region ranging up to about 10 kb and 12 kb upstream, respectively, were transfected into HFL cells, although the activity was significantly suppressed by $CoCl_2$. These results suggested that the mechanisms controlling CYP3A gene expression of HIF-1α chemical stabilizer in fetal hepatocytes might be different from those in adult hepatocytes, and that HIF-1α is not directly involved in regulation of CYP3A4 or CYP3A7 expression.

Keywords: CYP3A4; CYP3A7; cobalt chloride; human fetal liver cell; hypoxia; HIF-1α

Introduction

Cytochrome P450 (CYP) is a superfamily of hemoproteins that catalyze the oxidation of lipophilic substrates to more water-soluble products.[1] Among them, the human CYP3A subfamily includes four isoforms, CYP3A4, CYP3A5, CYP3A7, and CYP3A43.[2] The expression patterns of members of the CYP3A subfamily, especially CYP3A4 and CYP3A7, are characterized by marked interindividual variability and distinct developmental profiles. CYP3A4 is a major isoform that accounts for about 30% of the total amount of CYP in the adult human liver and is responsible for approximately 50% of the metabolism of known drugs by CYPs in human.[3,4] CYP3A7 is predominantly expressed in prenatal tissues, accounting for 50% of the total CYP content in the fetal liver.[5] CYP3A7 expression is detected as early as 50–60 days of gestation and persists until after birth, when it decreases over the first few months of life.[5,6]

Hypoxia activates transcription factors, which regulate the adaptive response of the cells to hypoxia.[7,8] Cells adapt to the low oxygen partial pressure by upregulating the transcription of multiple genes, such as vascular endothelial growth factor (VEGF), erythropoietin, and several glycolytic enzymes. Many molecular and cellular responses to hypoxia are mediated by the transcription factor hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimer consisting of a constitutively expressed subunit (HIF-1β) and an oxygen-regulated subunit (HIF-1α).[9] In the presence of oxygen and iron, HIF-1α is rapidly degraded *via* the prolyl-hydroxylase/von Hippel–Lindau pathway.[10] Hypoxia stabilizes HIF-1α, allowing formation of the heterodimer HIF-1α/β, which binds to hypoxia responsive elements (HRE) within the promoters of several genes involved in cell proliferation, differentiation, survival, cell migration, angiogenesis, and energy metabolism.[7,11] The hypoxic state can be mimicked

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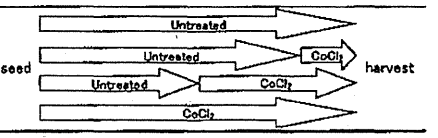
A

	$CoCl_2$ treatment (h)	Untreated (h)
U144→C0	0	144
U120→C24	24	120
U72→C72	72	72
U0→C144	144	0



B

	Hypoxia (h)	Normoxia (h)
N72→H0	0	72
N48→H24	24	48
N24→H48	48	24
N0→H72	72	0
H48→N24	48	24
H24→N48	24	48

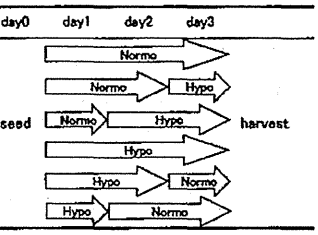


Fig. 1. Experimental protocols for (A) $CoCl_2$ treatment group and (B) hypoxia group

by chemical stabilizers of HIF-1α, such as cobalt chloride ($CoCl_2$) and the iron chelator deferoxamine.[12–16]

Early *in vivo* and *in vitro* studies suggested that acute hypoxia reduces the activity of drug metabolism.[17] Legendre *et al.* reported that drug-metabolizing enzymes, including CYP3A4, were downregulated by hypoxia and treatment with $CoCl_2$ in highly differentiated human hepatoma HepaRG cells.[18] They suggested that HIF-1α plays a central role in the repression of CYP3A4 in cells treated with $CoCl_2$.[18] We have reported previously that the expression mechanisms of CYP3A in human fetal liver (HFL) differ from those in the adult liver.[19] To our knowledge, the effects of HIF-1α chemical stabilizers and hypoxia on the expression of CYP3A enzymes in HFL have not been investigated, even at the mRNA level. The present study was performed to clarify the effects of HIF-1α stabilizer, $CoCl_2$ and hypoxia, and the involvement of HIF-1α on CYP3A4 and CYP3A7 expression in HFL cells.

Materials and Methods

Materials: Dulbecco's phosphate-buffered saline (PBS) was purchased from DS Pharma Biomedical Co. (Osaka, Japan). Penicillin-streptomycin-neomycin antibiotic mixture, SuperScript III First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR), and Lipofectamine 2000 were from Invitrogen Corp. (Carlsbad, CA). Williams' medium E, $CoCl_2$, and protease inhibitor cocktail were from Sigma-Aldrich Co. (St. Louis, MO). Cell Banker was from Wako Pure Chemicals (Osaka, Japan). Collagen (type I) was from Koken (Tokyo, Japan). PCR primers were purchased from Sigma Genosys (Hokkaido, Japan). SYBR Premix Ex Taq and LA PCR *in vitro* Cloning kit were obtained from TaKaRa Bio (Shiga, Japan). The Dual-Luciferase Reporter Assay System was obtained from Promega (Madison, WI). The Great EscAPe™ SEAP Chemiluminescence Kit 2.0 was from Clontech (Palo Alto, CA). The BCA Protein Assay Kit was from Thermo Fisher Scientific (Waltham, MA). All other reagents used were of the highest quality available.

Cells: HFL cells were obtained from Applied Cell Biology Research Institute (Kirkland, WA). Cell culture was initiated from a pool of six normal human liver tissues (average 13 weeks of gestation) by elutriation following dispase digestion of tissue. The cells were subcultured when the culture reached 90–100% confluence, and suspended in Cell Banker after proliferation. Aliquots of the suspensions were cryopreserved at −150°C until use.

Cell culture and $CoCl_2$ treatment: HFL cells were seeded onto dishes coated with type I collagen and cultured in Williams' medium E supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, and antibiotics (50 μg/mL penicillin, 50 μg/mL streptomycin, and 100 μg/mL neomycin) under an atmosphere of 5% CO_2 in air at 37°C. The medium was replaced daily with fresh medium. In the $CoCl_2$ treatment group, HFL cells were cultured for 144 h after seeding of 1×10^5 cells onto 6-well tissue culture plates (BD Biosciences, Heidelberg, Germany), and then treated with 200 μM $CoCl_2$ for 24 h, 72 h, and 144 h after cultivation for 120 h, 72 h, and 0 h (**Fig. 1A**). $CoCl_2$ was dissolved in sterile water and sterilized by membrane filtration. The $CoCl_2$ solution was added to culture medium at a ratio of one-thousandth to the medium. In the hypoxia groups, HFL cells were cultured for 72 h after seeding of 1×10^5 cells onto 6-well tissue culture plates, and then cultured after 72 h of normoxia (N) or normoxia followed by different periods (h) of hypoxygenation (N → H), at 72 h of hypoxia (H) or hypoxia followed by different periods (h) of reoxygenation (H → N) (**Fig. 1B**). The control group was cultured for 72 h under normoxic conditions. For hypoxia, HFL cells were incubated under atmospheric conditions of 3% O_2/5% CO_2/92% N_2 at 37°C.

RNA extraction and reverse transcription reaction: Total RNA was extracted from the treated cells using

Table 1. Sequences of primers and real-time PCR conditions

Primer name	Forward primer sequence (5′ to 3′)	Revers primer sequence (5′ to 3′)	Product size (bp)	Annealing temp.	Number of cycles	GenBank accession number
CYP3A4	CTGTGTGTTTCCAAGAGAAGTTAC	TGCATCAATTTCCTCCTGCAG	298	60	40	AF182273
CYP3A7	AGATTTAATCCATTAGATCCATTCG	AGGCGACCTTCTTTTATCTG	148	60	40	AF315325
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG	185	60	40	BC013310
VEGFA	TGCTTCTGAGTTGCCCAGGA	TGGTTTCAATGGTGTGAGGACATAG	176	60	40	NM001025366.1

an illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Buckinghamshire, UK). First-strand cDNA was generated from 4 μg of total RNA. The reverse transcription reaction was performed using the SuperScript III First-Strand Synthesis System for RT-PCR in accordance with the manufacturer's instructions (Invitrogen Corp.).

Real-time RT-PCR analysis: For detection of expression levels, CYP and VEGF mRNAs were analyzed by SYBR Green real-time quantitative RT-PCR. The levels of these mRNAs were normalized relative to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. All PCR procedures were performed using the 7300 Fast Real-time PCR System (Applied Biosystems) according to each manufacturer's instructions. PCR was performed using diluted cDNA template in 12.5-μL reaction mixtures containing 0.20 μM of each primer and 6.25 μL of SYBR Premix Ex Taq. The primers used are summarized in **Table 1**.

Western blotting analysis: HFL cells were lysed in lysate solution (20 mM Tris-HCl, pH 6.8, 50 mM NaCl, 10% glycerol, and 1% Triton X-100) supplemented with protease inhibitor cocktail after rinsing in Dulbecco's PBS. Western blotting analysis was carried out according to the method reported previously.[20] The proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred onto Immun-Blot PVDF Membranes (Bio-Rad, Hercules, CA). The blotted membrane was probed with anti-HIF-1α mouse monoclonal antibody (BD Transduction Laboratories, San Jose, CA). The immunoreactive proteins were visualized using ECL Plus Western Blotting Detection Reagents (GE Healthcare) and light emission was quantified with an Image Reader LAS-3000 (Fujifilm, Tokyo, Japan).

Luciferase and secreted alkaline phosphatase assays: The respective promoter fragments were linked to the firefly luciferase reporter gene in the pGL3-basic vector (Promega) or the secreted alkaline phosphatase (SEAP) reporter gene in the pSEAP2-basic vector (Clontech, Palo Alto) to produce reporter constructs. The *Renilla* luciferase gene (Promega) under the control of the thymidine kinase promoter (phRL-TK) was used as an internal control for firefly luciferase reporter gene expression. The 5′ untranslated region of the *CYP3A4* gene was isolated by PCR from a human genomic DNA library. The promoter region of the *CYP3A4* gene was prepared using 5′-ACGCGTGATGAACACATGCTACAGAATGGATAGC-3′ and 5′-CTCGAGTGCACAGCAGTGATTCAGTGAGG-3′ with *Mlu*I and *Xho*I restriction sites at the 3′-ends of the primers, respectively. The PCR product was digested with *Mlu*I and *Xho*I, and inserted into the *Mlu*I and *Xho*I sites of the pSEAP2-basic vector. The final product was named p3A4-10k (including from −9,532 to +12 bp). The 5′ untranslated region of the *CYP3A7* gene was isolated by PCR from a human genomic DNA library. The promoter region of the *CYP3A7* gene was prepared using 5′-ACGCGTGCTTGTTATCTGTGTACATGAGAGTC-3′ and 5′-GTCGACTGCACAGCAGTGATTCAGTGAGG-3′ with *Mlu*I and *Sal*I restriction sites at the 3′-ends of the primers, respectively. The PCR product was digested with *Mlu*I and *Sal*I and inserted into the *Mlu*I and *Sal*I sites of the pGL3-basic vector. The final product was named p3A7-12k (including from −12,688 to +13 bp). PCR was performed using an LA PCR *in vitro* Cloning kit according to the manufacturer's instructions (TaKaRa Bio).

HFL cells were transfected with pSEAP2-basic vector or the reporter plasmid p3A4-10k. In evaluation of CYP3A7 promoter activity, HFL cells were cotransfected with pGL3-basic vector and control vector phRL-TK or the reporter plasmid p3A7-12k using Lipofectamine 2000 in complete medium. After 4–6 h, medium was replaced with fresh medium, and the cells were exposed to $CoCl_2$ (200 μM) or hypoxia (3% O_2) for 48 h. After the specified exposure times, the cells transfected with pGL3-basic or p3A7-12k vector plasmids were harvested in 10 mM PBS (pH 7.5) containing 0.5 mM EDTA and frozen for subsequent assays. The reporter assay was performed using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega). After assay, the ratio of firefly luciferase activity to *Renilla* luciferase activity (relative luciferase activity) was used as the normalized luciferase activity. SEAP activity in culture medium was measured using a Great EscAPe™ SEAP Chemiluminescence Kit 2.0. The luminescence obtained from reactions of the luciferase and SEAP assays was then monitored using a Sirius Luminometer (Berthold, Pforzheim, Germany) and Glomax™ 96 Microplate Luminometer (Promega), respectively. SEAP activity was normalized relative to protein concentration.

Protein concentration: Protein concentration was determined using a BCA protein Assay Kit in accordance with the manufacturer's instructions, using bovine serum albumin as a standard.

Statistical analysis: All values are expressed as means ± standard deviation (SD). All data were analyzed by the unpaired Student's *t* test or Dunnett's multiple compari-

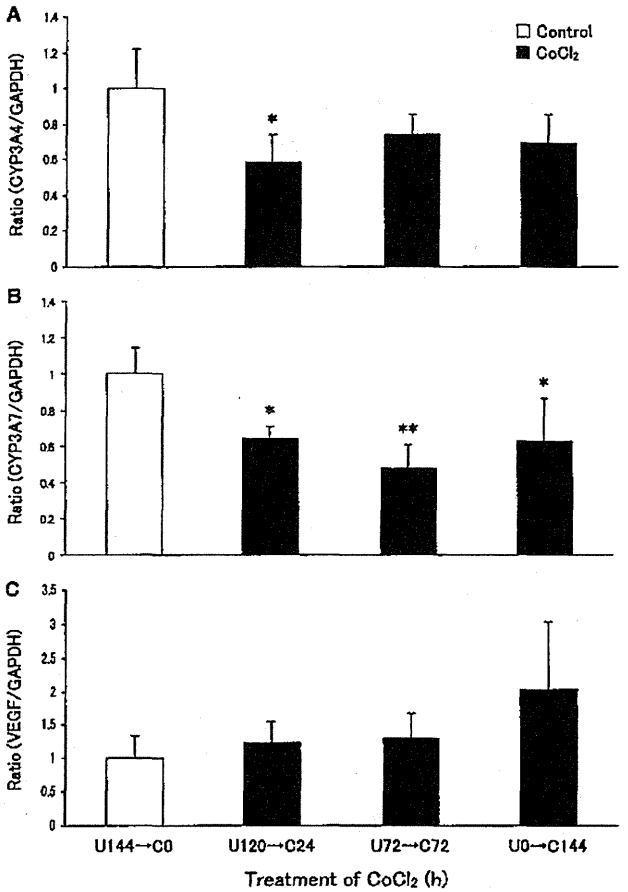


Fig. 2. Effects of $CoCl_2$ on expression of CYP3As and VEGF mRNA in HFL cells

(A) CYP3A4 mRNA level, (B) CYP3A7 mRNA level, (C) VEGF mRNA level. HFL cells were treated with $CoCl_2$ (200 μM) for 24 h (U120 → D24), 72 h (U72 → D72), and 144 h (U0 → D144), after cultivation for 120 h, 72 h, and 0 h, respectively. Total RNA was extracted from HFL cells cultured for 144 h. The levels of expression of CYP3As and VEGF mRNAs were analyzed by SYBR Green real-time RT-PCR as described in the Materials and Methods. Data are presented as the ratios of CYP3As and VEGF to GAPDH and normalized relative to 1.0 for the untreated group. Values are expressed as the means ± SD ($n = 3$). Significantly different from control (*$p < 0.05$, **$p < 0.01$).

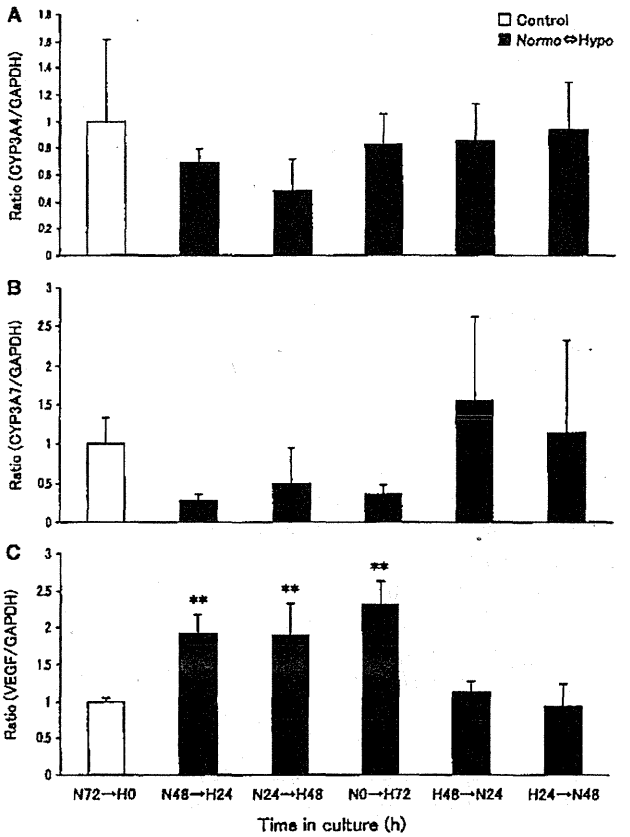


Fig. 3. Effects of hypoxia on expression of CYP3As and VEGF mRNA in HFL cells

(A) CYP3A4 mRNA level, (B) CYP3A7 mRNA level, (C) VEGF mRNA level. HFL cells cultured for 72 h of normoxia (N) or normoxia followed by different periods (h) of hypoxygenation (N → H), at 72 h of hypoxia (H) or hypoxia followed by different periods (h) of reoxygenation (H → N). Total RNA was extracted from HFL cells cultured for 72 h. CYP3As and VEGF mRNA expression were analyzed by SYBR Green real-time RT-PCR as described in the Materials and Methods. Data are presented as the ratios of CYP3As and VEGF to GAPDH and normalized relative to 1.0 for normoxia. Values are expressed as the means ± SD ($n = 3$–5). Significantly different from control (**$p < 0.01$).

son test for significance of differences between the mean values for each group. In all analyses, $p < 0.05$ was taken to indicate statistical significance. All experiments were repeated more than three times to confirm reproducibility.

Results

Effects of $CoCl_2$ on expression of CYP3As and VEGF mRNAs: To evaluate the effects of HIF-1α chemical stabilizer on the expression of CYP3A4 and CYP3A7 mRNAs in HFL cells, the cells were treated with $CoCl_2$ (200 μM) for 24, 72, and 144 h. VEGF mRNA expression was monitored as a marker of HIF-1α accumulation in cells, because VEGF gene expression was induced by an increase in HIF-1α level. As shown in **Figure 2**, the levels of CYP3A4 and CYP3A7 mRNA expression in HFL cells were decreased by 59% and 48%, respectively, compared to the control by treatment with $CoCl_2$ for 24 h and 72 h, although the level of VEGF mRNA expression was not significantly altered by this compound.

Effects of hypoxia on expression of CYP3As and VEGF mRNAs: To evaluate the effects of hypoxia on the expression of CYP3A4, CYP3A7, and VEGF mRNAs in HFL cells, the cells were cultured under 3% O_2 for 72 h. As shown in **Figure 3**, the expression levels of CYP3A4 and CYP3A7 mRNAs tended to be reduced by hypoxia. In contrast, the level of VEGF mRNA expression was increased

by about 2-fold in HFL cells cultured under normoxic conditions for 48 h followed by hypoxia for 24 h, normoxia for 24 h followed by hypoxia for 48 h and hypoxia for 72 h, although the level remained unchanged in HFL cells cultured under hypoxia for 48 h followed by normoxia for 24 h or hypoxia for 24 h followed by normoxia for 48 h (**Fig. 3C**).

Accumulation of HIF-1α by $CoCl_2$ treatment or hypoxia: To confirm whether HIF-1α protein was accumulated by $CoCl_2$ or hypoxia, we harvested the lysates of HFL cells cultured in the presence of 200 μM $CoCl_2$ or under 3% O_2 for 24 and 72 h, and subjected them to Western blotting analyses (**Fig. 4**). A protein immunologically related to HIF-1α was detected. When HFL cells were cultured in medium containing $CoCl_2$ for 72 h, the contents of HIF-1α protein were significantly increased by 3.3-fold compared to controls, although the protein level was not changed by $CoCl_2$ treatment for 24 h. The contents of HIF-1α protein in HFL cells were significantly increased by 2.7-fold and 2.1-fold, respectively, compared to control by hypoxia for 24 h and 72 h.

Transcriptional activities of *CYP3A4* and *CYP3A7* genes: The SEAP reporter plasmid containing the CYP3A4 promoter up to about −10 kb (p3A4-10k) and the luciferase reporter plasmid containing the CYP3A7 promoter up to about −12 kb (p3A7-12k) were transfected into HFL cells treated with 200 μM $CoCl_2$ and cultivated under 3% O_2 (**Fig. 5**). There were no significant changes in either reporter activity with 3% O_2. On the other hand, $CoCl_2$ significantly suppressed the reporter activities of both p3A4-10k and p3A7-12k.

Discussion

Legendre *et al.* reported that $CoCl_2$, hypoxia, or HIF-1α overexpression led to CYP3A4 downregulation in HepaRG cells.[18] They suggested that HIF-1α was one of the most important factors in CYP3A4 repression and might regulate CYP3A4 expression by an indirect mechanism or by interacting with a DNA regulatory sequence localized somewhere else in the CYP3A4 gene.[18] In the present study, the effects of $CoCl_2$ on CYP3A4 and CYP3A7 mRNAs in HFL cells were observed and compared to those seen under hypoxic conditions. The expression levels of CYP3A4 and CYP3A7 mRNAs were significantly decreased by $CoCl_2$, although hypoxia tended to decrease these expression levels.

VEGF induction has been demonstrated in endothelial cells exposed to cobalt and hypoxia.[21–24] It has been reported that the upregulation of VEGF expression is mediated through an increase in HIF-1α level.[24] Immuno-

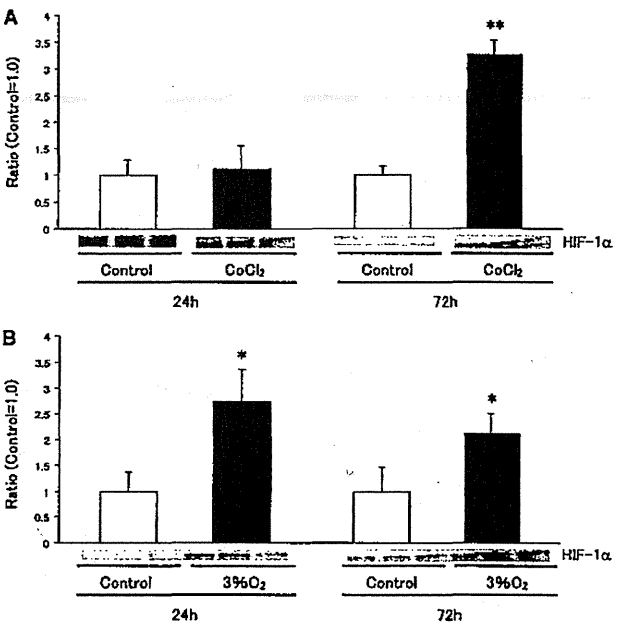


Fig. 4. Western blotting analysis of HIF-1α in HFL cells treated with $CoCl_2$ and hypoxia
HFL cells treated with $CoCl_2$ (A) and cultured in 3% O_2 (B) for 24 h and 72 h after seeding were subjected to SDS-PAGE and electrophoretically transferred onto membranes. The proteins were reacted with anti-HIF-1α mouse monoclonal antibody. Each lane contained 45 μg of protein. Values are expressed as the means ± SD ($n = 3$). Significantly different from control (*$p < 0.05$, **$p < 0.01$).

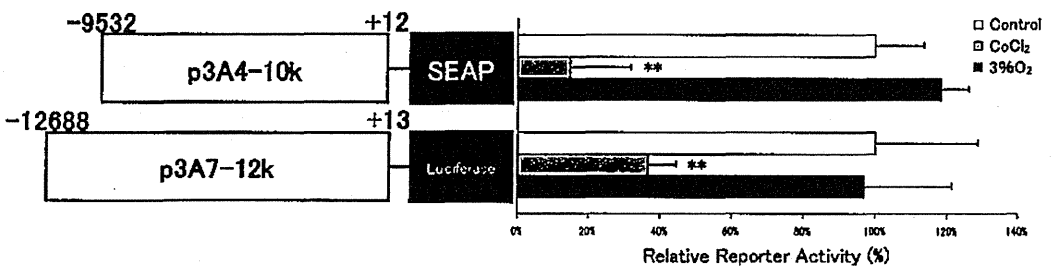


Fig. 5. Transcriptional activities of CYP3A4 and CYP3A7 in HFL cells
HFL cells were transfected with pSEAP2-basic vector or the reporter plasmid p3A4-10k. In the evaluation of CYP3A7 promoter activity, HFL cells were cotransfected with pGL3-basic vector and control vector phRL-TK or the reporter plasmid p3A7-12k. After 4–6 h, medium was replaced with fresh medium, and the cells were exposed to $CoCl_2$ (200 μM) or 3% O_2 for 48 h. The ratio of firefly luciferase activity to *Renilla* luciferase activity was used as the normalized luciferase activity. SEAP activity was normalized relative to protein concentration. Values are expressed as the means ± SD ($n = 8–12$). Significantly different from control (**$p < 0.01$).

logically related proteins detected by the antibody raised against human HIF-1α were detected in HFL cells on Western blotting analysis (**Fig. 4**). We have confirmed that the effects of treatment with 200 μM $CoCl_2$ for 24 h and 72 h on CYP3A4 and CYP3A7 were almost the same as those of 200 μM $CoCl_2$ for 24 h and 72 h, respectively, after cultivation for 120 h and 72 h (data not shown). The content of HIF-1α was increased by 2–3-fold compared to controls by $CoCl_2$ and hypoxia (**Fig. 4**). The expression of VEGF mRNA was induced by $CoCl_2$ and hypoxia in HFL cells, although the change in the cells treated with $CoCl_2$ was not significant (**Figs. 2C** and **3C**). VEGF mRNA levels may not be any more susceptible to HIF-1α chemical stabilizers than HIF-1α protein levels. These results of $CoCl_2$ and hypoxia on HIF-1α protein were consistent with the expression patterns of VEGF mRNA in HFL cells. HIF-1α is rapidly degraded under normoxia, as oxygen-dependent HIF-1α stabilization requires an oxygen tension of <2–3% O_2.[25,26] The switch back to normoxia from hypoxia caused rapid degradation of HIF-1α, even if it had been accumulated in HFL cells by cultivation under hypoxia. The switch back to normoxia from hypoxia caused no obvious changes in VEGF mRNA level compared with controls incubated for 72 h under normoxic conditions.

HIF-1 DNA binding activity is induced when mammalian cells are subjected to hypoxia.[27,28] We searched for the consensus sequence of HRE in the promoter regions of CYP3A4 and CYP3A7 up to 10 kb and 12 kb upstream of the respective transcriptional start codons and identified 6 and 7 canonical mammalian HREs, respectively, in the promoter regions of CYP3A4 and CYP3A7. However, the transcriptional activities of CYP3A4 and CYP3A7 were not decreased by hypoxia when the reporter plasmids containing the promoter region up to about 10 kb and 12 kb upstream, respectively, were transfected into HFL cells (**Fig. 5**). These results indicated that HIF-1α is not directly involved in the hypoxia-induced downregulation of CYP3A7. On the other hand, the transcriptional activities of *CYP3A4* and *CYP3A7* genes were significantly suppressed by $CoCl_2$. These results were consistent with the effects of $CoCl_2$ on the expression of CYP3A4 and CYP3A7 mRNAs. However, we could not clarify the mechanisms underlying the suppressive effects of $CoCl_2$ on the expression of CYP3A4 and CYP3A7 in HFL cells.

The results of the present study indicated that the expression levels of CYP3A4 and CYP3A7 mRNAs were significantly decreased by $CoCl_2$ in HFL cells. The content of HIF-1α was increased by $CoCl_2$ and hypoxia. In conclusion, these results suggest that the mechanisms by which the HIF-1α chemical stabilizer controls CYP3A gene expression in fetal hepatocytes may be different from those in adult hepatocytes, and that HIF-1α is not directly involved in regulation of CYP3A4 and CYP3A7 expression.

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