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G.知的財産権の出願・登録状況

現在までに出願、登録はない。

厚生科学研究費補助金（萌芽的先端医療技術推進研究事業）

分担研究報告書

ヒト胎盤細胞を用いた新治療開発のための基礎的検討

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研究要旨

満期産のヒト胎盤から得られた間葉系細胞を培養して検討したところ、血管新生因子(hVEGF)以外に、塩基性線維芽細胞成長因子(bFGF)やアンギオポイエチンも産生していることが明らかとなった。他の細胞の産生する成長因子の組み合わせと比較すると、癌細胞とは類似せず正常線維芽細胞のパターンと類似していた。これは胎盤でらせん動脈という正常血管が形成されることと一致する。ヒト胎盤由来間葉系細胞の溶解液を親水軟膏にまぜ、マウスの皮膚損傷モデルに投与すると有意に創傷治癒が促進された。この事実は細胞移植以外にもこの細胞を使った治療法がある可能性を示唆する。

A.研究目的

胎盤は母体と胎児との栄養交換を行う臓器であり、胎児の成長とともに短期間で発達する。またその機能を果たすため非常に血管に富んだ臓器であることはよく知られている。このような事実から、我々は胎盤を構成する細胞は豊富に血管新生因子が産生するのではないかと考え、ヒト胎盤を酵素的に処理して培養し、その上清中あるいは細胞抽出物中のヒト血管内皮成長因子（human vascular endothelial growth factor: hVEGF）を測定した。その結果、胎盤由来間葉系細胞(human placenta-derived mesenchymal cells: hPDMC)が多量のhVEGFを産生することを見い出した。しかしながらhPDMCから分泌されたhVEGFの生物活性、hPDMCの細胞移植によるin vivoでの血管新生、hPDMCから分泌されるhVEGFの薬物動態、などについては不明であった。昨年度は、①hPDMCから産生されるhVEGFの生物活性の検討、②虚血動物モデルでのhPDMCの細胞移植びよる血流の改善、③細胞移植されたhPDMCからhVEGFの分泌動態、の３つを検討し、hPDMCの培養上清はhUVEC (human umbilical vein endothelial cell)の増殖を刺激し、hVEGFに生物活性があることが示された。また細胞移植はマウスの虚血を改善し、real-time RT-PCRで検索したhVEGF　mRNAレベルから少なくとも移植後７日間は局所でhVEGFを産生していることが明らかとなった。本年度はhPDMCの他の性質を調べ、臨床応用への可能性を検索する。

B.研究方法

① hPDMCから産生されるhVEGF以外の物質の検討

hPDMCが産生するhVEGF以外の血管新生因子について検討するために、hPDMCのmRNAを抽出し、塩基性線維芽細胞成長因子(bFGF)、アンギオポイエチン１，アンギオポイエチン２、血小板由来成長因子(PDG

F)のRT-PCRを行う。これで検出できる因子については、培養上清でELISAを用いて蛋白濃度を測定する。

②hPDMC細胞溶解液の創傷治癒への効果

hPDMCの細胞溶解液を親水軟膏に混合しマウスの創傷モデルに投与する。麻酔下でマウスの背側の皮膚を2カ所(左背側と右背側)それぞれ15mm角に切り取り、1方にhPDMC溶解液を含む親水軟膏を塗布し、対側に対照の軟膏を塗布する。30分間静置し、成長因子が傷害部に十分作用するようにする。創傷が治癒する約10日後に至るまで、72時間毎にマウスを麻酔し、デジタルカメラで皮膚欠損部位を撮影する。コンピューターを用いて画像から皮膚欠損部の面積を計算し、対照と比較する。

C.研究結果

① hPDMC から産生される hVEGF 以外の物質の検討

RT-PCR の結果、bFGF とアンギオポイエチン 1 mRNA の発現があることが判明した。ELISA を用いて bFGF を測定したところ、細胞培養上清中には検出されなかったが、細胞溶解液に高濃度に存在することが明らかとなった。アンギオポイエチン 1 についてはよい ELISA が内ので今後の検討課題となった。

②hPDMC細胞溶解液の創傷治癒への効果

皮膚切除 10 日後に計測した欠損皮膚は、対照群で 99.1±11.1mm²(mean±SE、n=20)に対し hPDMC 投与群では 70.5±6.4mm² であった。hPDMC 投与群は対照群に比較して有意に皮膚損傷を改善した(Mann-Whitney u-test, $p<0.05$)。

D.考察

昨年度までに hPDMC の細胞移植がマウスの下肢虚血モデルで血流を改善することを示したが、細胞移植は手間のかかる治療であり、可能であればより簡便な治療を開発する必要がある。また hPDMC からは hVEGF 以外の血管新生因子や成長因子が産生されている可能性があったため、本研究を発案した。その結果 bFGF やアンギオポイエチン 1 も産生していることが明らかとなった。これらの事実から、hPDMC の溶解液を含む軟膏が開発できるのではないかと考え、親水軟膏に溶解液を混合してマウス皮膚損傷モデルに投与したところ、治癒の促進が認められた。今回はマウスの皮膚切除モデルを用いたが、マウスの火傷モデル、褥瘡モデルでも効果があるかを検討する予定である。

E.結論

hPDMC は hVEGF 以外にも bFGF やアンギオポイエチン 1 を産生し、hPDMC の溶解液を含む軟膏は、マウス皮膚損傷モデルの治癒を促進させた。

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2. 学会発表

なし

G.知的財産権の出願・登録状況

（予定を含む）

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

厚生労働科学研究費補助金（萌芽的先端医療技術推進研究事業）
分担研究報告書

薬物トランスポーターの遺伝子発現多様性に関する研究

分担研究者　油谷浩幸　東京大学国際・産学共同研究センター　教授

研究要旨
薬物トランスポーターの遺伝子発現量の多様性について、EBV 不死化リンパ球 RNA をマイクロアレイ解析することにより検討した。また、単塩基多型を利用して発現量多様性を質量分析測定装置による測定を開発すると共にトランスポーター遺伝子の 2 つのアレル間の発現量多様性があることを示した。

A.研究目的

トランスポーター遺伝子の遺伝子多型と血中薬剤濃度との関連についての報告が近年散見されるが、遺伝子多型には蛋白質配列の変異を伴わないものも多くその意義付けはなかなかに困難である。本研究においてはアレル特異的な遺伝子発現の多様性の有無とともに発現量の多様性を制御する DNA 多型について解析することを目的とする。本年は発現量多様性解析法の開発とトランスポーター遺伝子の発現量多様性の検討を行った。

B.研究方法

1. ABC トランスポーター遺伝子の発現量多様性

単塩基多型情報を用いてアレル間の発現量の違いを検出することができる。多型部位を含む DNA 配列をシークエンサーを用いて配列決定すれば、それぞれのアレルからのシグナルが 1:1 で検出される。cDNA 配列中に存在する多型を利用してそれぞれのアレルから読まれる RNA 量を比較することが出きる。また、SNP 検出用マイクロアレイをもちいてトランスポーター遺伝子アレル間の発現量比について検討した。 試料となる DNA および cDNA を制限酵素 XbaI あるいは HindIII で切断し、アダプターをつけて、そのアダプターに特異的なプライマー (XbaI あるいは HindIII についてそれぞれ 1 種類ずつ) を用いて PCR 反応により増幅し、Biotin 標識を行う。 2 枚の SNP 検出用マイクロアレイは各 SNP のアレルごとに相補的になるように設計されており、ハイブリダイゼーション後、シグナルに基づいて試料の SNP を判定し、また各アレルの発現量をシグナル強度又はシグナル比に基づいて比較することができる。

不死化リンパ球から抽出した DNA および RNA を用いて上記実験を行った。

2. 質量分析による遺伝子発現量多様性解析法の開発

単塩基多型（SNP）に由来する質量の差を利用してアレルを識別することにより、アレル間の遺伝子発現の変化を定量的に検出する方法を開発した。DNA を鋳型としてプライマーエクステンションを行う際に SNP 部位を含めれば、産生される配列の質量を比較することによりゲノタイピングを行うことが可能である。一方、cDNA を鋳型として同様の反応を行うことにより、RNA 量のアレル間の違いを検出することが可能である。

SNP 部位を含めてプライマーエクステンションし、ddNTP を取り込ませることにより伸長反応を停止させる。産物をマトリックス上にスポットした後に飛行時間型 MS（Sequenom）によりそれぞれのアレルからの産生物を定量した。

(倫理面への配慮)

本年度の研究計画では臨床情報を有していないDNAおよびRNA試料についてゲノタイピングを施行した。なおEBVにより不死化したリンパ球株はATCCより購入したものであり、連結不能匿名化されている。本研究は東京大学先端科学技術研究センター研究倫理審査委員会の承認を受けている。

C.研究結果

1.ABCトランスポーター遺伝子の発現量多様性

リンパ球においてアレル間の遺伝子発現量が3倍以上異なるトランスポーター遺伝子が同定された。

2.質量分析による遺伝子発現量多様性解析法の開発

DNAを鋳型として測定した場合にはアレル間のシグナルの比率はほぼ1:1であった。一方、cDNAを鋳型とした場合にはアレル間の発現が10倍以上異なるサンプルも存在した。

D.考察

本研究事業ではトランスポーター遺伝子の発現多様性に注目して研究を進めてきた。不死化リンパ球RNAの解析では発現量に大きなばらつきが認められるABCトランスポーター遺伝子が認められた。同一家系からの検体を解析することにより、発現量多様性が遺伝性のものであるのか否かについて今後明らかにされるものと期待される。また、本年はリンパ球あるいは末梢血を用いて解析を行ったが、腸管や肝臓などの薬物動態を制御する主要臓器や腫瘍細胞などでの発現にもジェノタイプによる発現量の違いがあるかどうかについて同様な検討を進める必要がある。

トランスポーター遺伝子が多数のエクソンから構成されることからスプライシング変異が多様な転写産物をもたらす。次年度以降にはゲノムタイリングアレイを用いて変異転写産物の多様性についての検討も行う予定である。

E.結論

SNPを利用してトランスポーター遺伝子の発現量多様性を検出できた。質量分析法を用いることによりアレル間の遺伝子発現量の変動を検出することに成功した。

F.健康危険情報

なし

G.研究発表

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3. 第14回難病治療研究会（東京）7/8 アレイを用いた機能ゲノム解析
4. LSBM国際シンポジウム（東京）7/13 2nd International Symposium on New Frontiers of Systems Biology and Medicine Novel Biomarker discovery through cancer genomics
5. 第11回箱根肝臓シンポジウム(箱根) 7/22 肝発癌におけるゲノム変異の統合的解析
6. BioJapan2004シンポジウム「ゲノム・プロテオーム解析の癌診療へのインパクト」(東京) 9/28 **ゲノム解析の癌研究への応用**
7. 第11回群馬clinical Oncology Research勉強会（前橋）11/11 アレイ解析技術と癌研究
8. 三菱化学ヘルスケアフォーラム（東京）11/13 **癌の分子診断と治療への展開**
9. 国際疲労学会（軽井沢）2/11 International Conference on Fatigue Science 2005 Gene Expression Signatures in CFS patients
10. 東京大学国際シンポジウム（東京）2/18 The University of Tokyo International Symposium – Frontiers in Drug Development. Genomic Technology in Drug Development
11. 第13回広島大学・がんセミナー学術講演会（広島）3/1アレイ解析によるhigh throughput biology
12. 第 7 回 Tokyo Urological Research Conference (TURC) 東京、3/5 がんゲノム情報の網羅的解析

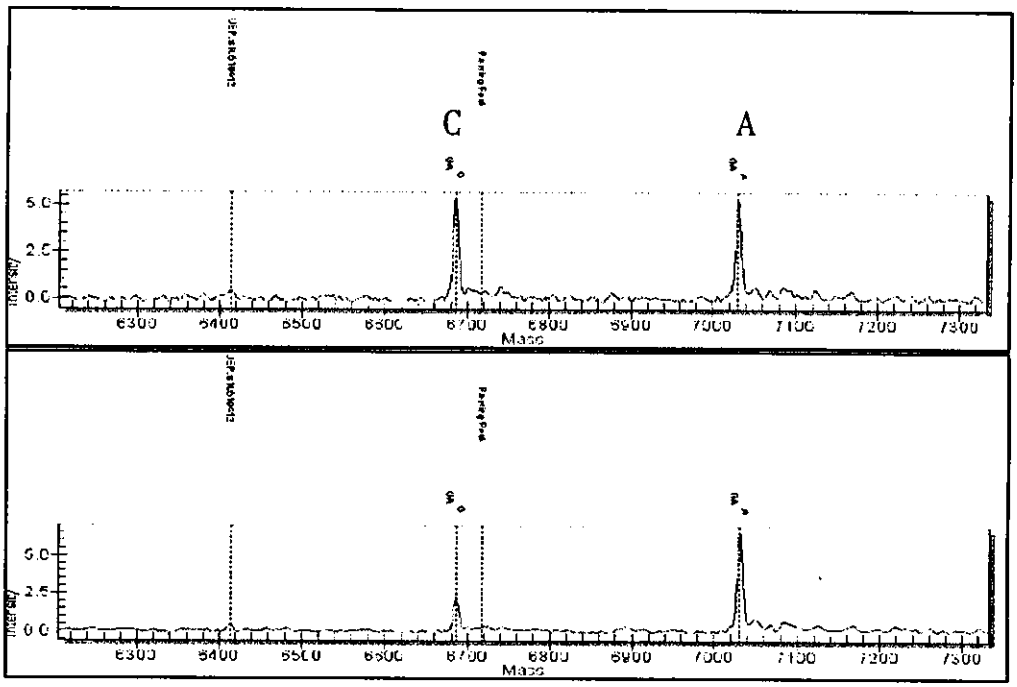
H.知的財産権の出願・登録状況(予定も含む)

1. 特許取得
   なし
2. 実用新案登録
   なし
3. その他
   なし

図　質量分析によるアレル間発現量多様性の検出

genomic

cDNA



研究成果の刊行に関する一覧表

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IV. 研究成果の刊行物・別刷り

Functional analysis of single nucleotide polymorphisms of hepatic organic anion transporter OATP1B1 (OATP-C)

Megumi Iwai[a], Hiroshi Suzuki[a], Ichiro Ieiri[b], Kenji Otsubo[b] and Yuichi Sugiyama[a]

Objective **Two kinds of single nucleotide polymorphism (SNP; Asn130Asp and Val174Ala) are frequently observed in the liver specific transporter, organic anion transporting polypeptide 1B1 (OATP1B1/OATP-C) gene. Although these two SNPs occur independently in European-Americans, Val174Ala is mostly associated with Asn130Asp in Japanese. Our previous in-vivo studies in Japanese subjects indicated that the non-renal clearance of pravastatin was decreased to 13% of that in wild-type subjects (Nishizato *et al. Clin Pharmacol Ther* 2003;73(6):554–564). The purpose of the present study is to characterize the function of SNPs variants of OATP1B1 in cDNA transfected cells.**

Methods **The localization and transport activity were analyzed in HEK293 cells stably expressing wild-type OATP1B1 (OATP1B1*1a), OATP1B1*1b (Asn130Asp), OATP1B1*5 (Val174Ala) and OATP1B1*15 (Asn130Asp and Val174Ala). To characterize the intrinsic V_{max}, observed V_{max} in uptake study were normalized by the expression level estimated from Western blotting.**

Results **All SNP variants are predominantly located on the cell surface. No significant alteration was observed in K_m values for the transport of 17β-estradiol 17β-D-glucuronide ($E_2$17βG), a typical substrate of OATP1B1, among these SNP variants. However, the normalized V_{max} value for OATP1B1*15 was drastically decreased to less than 30% compared with OATP1B1*1a. In contrast, the transport activity of OATP1B1*1b (Asn130Asp) and OATP1B1*5 (Val 174Ala) was similar to that of OATP1B1*1a.**

Conclusions **These results are consistent with the results of our previous clinical studies. It is thus suggested that in-vivo disposition may be predicted from in-vitro results using recombinant transporters. *Pharmacogenetics* 14:749–757 © 2004 Lippincott Williams & Wilkins**

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Keywords: OATP1B1, SNPs, haplotype, hepatic transport, organic anion

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Introduction

The administration of the same amount of certain drugs to individuals often results in variability in drug disposition. This is partly due to the presence of hereditary differences, such as single nucleotide polymorphisms (SNPs), in the genes encoding drug metabolizing enzymes and/or transporters [1,2]. Although the correlation between genotype and phenotype has been studied extensively as far as drug metabolizing enzymes are concerned [3,4], only limited information is available on the SNPs of the genes of drug transporters which are responsible for drug disposition [5].

Some pieces of information are available on organic anion transporting polypeptide 1B1 (OATP1B1/OATP-C), a basolaterally located transporter, which is responsible for the hepatic uptake of a series of organic anions, including 17β-estradiol 17β-D-glucuronide ($E_2$17βG), estrone-3-sulfate (E_1S), taurocholate, conjugated bilirubin, and anionic drugs such as pravastatin, from the portal vein to hepatocytes [6–11].

At present, several kinds of SNP variants have been identified in the human OATP1B1 gene. As far as the nomenclature of OATP1B1 variants is concerned, the cDNA sequence reported by Konig *et al.* [7] is designated as OATP1B1*1a. Recently, using stably transfected MDCKII cells, Michalski *et al.* [12] have identified a naturally occurring mutation in the OATP1B1 gene leading to an impairment of the protein mutation with reduced localization and abolished transport activity. The missorting to the membrane surface was also confirmed by intracellular localization of the mutant protein in the cryosections of human liver [12]. Tirona *et al.* [13] have identified the SNPs in the OATP1B1 gene in European-American

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and African-American subjects, and examined their function by transfecting the cDNAs into HeLa cells. Although they found that the expression levels of OATP1B1*3 (Val62Ala), OATP1B1*5 (Val174Ala), OATP1B1*6 (Ile353Thr) and OATP1B1*9 (Gly488Ala) were similar to that of OATP1B1*1a by Western blot analysis of cell lysate, their degree of expression on the cell surface was reduced and consequently, a reduction in the transport of $E_2$17βG and E_1S has been observed [13]. In contrast, using HEK293 cells transiently expressing OATP1B1, Nozawa *et al.* [14] reported that the cellular localization and transport function of OATP1B1*5 were no different from that of OATP1B1*1a, when E_1S is used as a ligand. The cells used to determine the function of OATP1B1 were different in the two reports, and it remains to be clarified whether the function of OATP1B1*5 is similar to that of OATP1B1*1a [14].

Recently, Nozawa *et al.* [14] found a novel OATP1B1 allele, OATP1B1*15, possessing both Asn130Asp and Val174Ala variants in Japanese subjects. OATP1B1*15 is different from OATP1B1*1b which is associated with Asn130Asp, but not with Val174Ala, and OATP1B1*5 which is associated with Val174Ala, but not with Asn130Asp. Although the allelic frequency of OATP1B1*5 is 14% in European-Americans, no Japanese subjects with this allele were found in our previous study [15]. In contrast, most of the Japanese subjects who have Val174Ala also have Asn130Asp and, therefore, a clear ethnic difference was observed in the frequency of the haplotype. However, no information is available on the in-vitro function of OATP1B1*15. To investigate the effect of SNPs on drug disposition, we recently reported the disposition of pravastatin, an HMG-CoA reductase inhibitor which is taken up by OATP1B1, in healthy Japanese subjects [15]. We found that the non-renal clearance of pravastatin in healthy Japanese subjects with the OATP1B1*15 allele is reduced to 13% of the wild-type (OATP1B1*1a). However, in-vitro evidence to support this in-vivo result is still lacking. In the present study, we constructed HEK293 cells stably expressing OATP1B1*1a, OATP1B1*1b, OATP1B1*5 and OATP1B1*15 and characterized their cellular localization and transport activity.

Material and methods

Materials

[^{3}H] $E_2$17βG (45.0 μCi/nmol) and [^{3}H] E_1S (46.0 μCi/nmol) were purchased from New England Nuclear (Boston, MA, USA). All other chemicals were commercially available and of reagent grade.

Construction of OATP1B1 SNP variants expressed in HEK293 cells

Human OATP1B1*1b (Asn130Asp) cDNA was subcloned into pcDNA3.1 (+) (Zeocin) (Invitrogen, Carlsbad, CA, USA). To construct the other SNP variants, point mutations were introduced by using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The introduction of the mutations was verified by full sequencing. Wild-type and SNP variants of OATP1B1 in pcDNA3.1 were transfected to HEK293 cells grown on a 12-well plate with Fugene 6 (Roche Diagnostics Corporation, Indianapolis, IN, USA) according to the manufacturer's instructions. Then, HEK293 cells were selected by 200 μg/ml Zeocin and colonies were picked up and the colonies which had the highest transport activities were used in the functional analysis. HEK293 cells stably expressing OATP1B1 were cultured in low-glucose Dulbecco's modified Eagle's medium (GIBCO BRL, Gaithersburg, MD, USA) after addition of 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml), zeocin (200 μg/ml) (Invitrogen) at 37°C with 5% CO_2, and 95% humidity. Sodium butyrate (5 mM) was added to the medium 24 hr before all experiments to induce the expression of OATP1B1.

Immunocytochemical staining

HEK293 cells stably expressing OATP1B1 were grown on a poly-L-lysine coated cover glass (Micro cover glass, 18 × 18 mm and 0.12–0.17 mm thick, Matsunami Glass Ind., Osaka, Japan). After fixation in −20°C methanol for 10 min and permeabilization in 1% Triton-X in phosphate-buffered saline (PBS) for 10 min, cells were incubated with the polyclonal antibody against OATP1B1 [16] diluted 50-fold in PBS for 1 h, washed three times with PBS, and then incubated with goat anti-rabbit immunoglobulin G (IgG; Alexa 488, Molecular Probes, Inc., Eugene, OR, USA) diluted 250-fold in PBS for 1 h. The localization of OATP1B1 protein was visualized by confocal lazer microscopy (Zeiss LSM-510; Carl Zeiss Inc., Thornwood, NY, USA).

Cell surface biotinylation

HEK293 cells stably expressing OATP1B1s were grown on 24-well plates. Cells were washed with ice-cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-Ca/Mg), then incubated twice with NHS-SS-biotin (1.5 mg/ml; Pierce Biotechnology, Inc., Rockford, IL, USA) at 4°C for 20 min. Then, the cells were washed with PBS Ca^{2+}/Mg^{2+} containing glycine (100 mM) and incubated with the same buffer for 20 min at 4°C. After removing the buffer, cells were disrupted with 50 μl lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton-X-100, pH 7.5) containing 1% sodium dodecyl sulfate (SDS) and protease inhibitor (0.1 mM phenylmethylsulfonyl fluoride) at 4°C for 30 min. To reduce the SDS concentration, samples were diluted with 450 μl of lysis buffer. Then, 50 μl of streptavidin-agarose beads (Pierce) was added to the lysate, and incubated at 4°C overnight with end-over-end rotation. Following centri-

fugation, the beads were washed three times with lysis buffer, twice with high salt lysis buffer (50 mM Tris, 500 mM NaCl, 5 mM EDTA, 0.1% Triton-X-100, pH 7.5), and once with low salt lysis buffer (50 mM Tris, pH 7.5). The biotinylated proteins were released by incubation with 40 µl 3× SDS loading buffer (BioLabs, Hitchin, UK) diluted to 1× SDS with PBS for 5 min at 60°C. Samples for total cell lysates (15 µl) and biotinylated proteins (2 µl) were subjected to the Western blot analysis.

Western blotting

Membrane fractions were prepared from HEK293 cells stably expressing OATP1B1 as described previously [17]. These crude membrane fractions were diluted with 3× SDS loading buffer and separated on 7% SDS-polyacrylamide gel with a 4.4% stacking gel. Proteins were transferred electrophoretically to a nitrocellulose membrane (Millipore, Bedford, MA, USA) using a blotter (Bio-Rad Laboratories, Richmond, CA, USA) at 15 V for 1 h. The membrane was blocked with 2.5% skimmed milk for 1 h at room temperature. Then, the membrane was incubated for 1 hr at room temperature with 500-fold diluted anti-OATP1B1 rabbit serum. For the detection of OATP1B1, the membrane was allowed to bind to 5000-fold diluted horseradish peroxidase-labeled anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Amersham, UK) for 1 h at room temperature. The enzyme activity was assessed using ECL Plus Western blotting Starter Kit (Amersham Biosciences, Inc.) with luminescent image analyzer (LAS-1000 plus, Fuji Film). The molecular weight was determined using a prestained protein marker (New England BioLabs, Beverly, MA).

Transport studies

Transport studies of [^{3}H] $E_2$17βG and [^{3}H] E_1S were carried out as described previously [18]. Uptake was initiated after cells were washed twice and preincubated with Krebs–Henseleit buffer at 37°C for 15 min. The Krebs–Henseleit buffer consists of 118 mM NaCl, 23.8 mM $NaHCO_3$, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM $CaCl_2$, adjusted to pH 7.4. After the removal of the incubation buffer, the uptake was terminated at designed times by adding ice-cold Krebs–Henseleit buffer, dissolved in 500 µl 0.2 N NaOH, and kept overnight. Aliquots (450 µl) were transferred to scintillation vials after adding 100 µl 1 N HCl. The radioactivity associated with the cells and medium was determined in a liquid scintillation counter after addition of 2 ml scintillation fluid (NACALAI TESQUE, Kyoto, Japan). The remaining 50 µl of the aliquots of the cell lysate was used to determine the protein concentration by the method of Lowry with bovine serum albumin as a standard.

Ligand uptake is given as the volume of distribution (µl/mg of protein) determined as the amount of ligand associated with the cells (pmol/mg of protein) divided by the medium concentration (pmol/µl). Specific uptake was obtained by subtracting the uptake into vector-transfected cells from the uptake into cDNA-transfected cells. Kinetic parameters were obtained using the following equation.

$$v = \frac{V_{max} \times S}{K_m + S} + P_{dif} \times S$$

where v is the uptake velocity of the substrate (pmol/min/mg of protein), S is the substrate concentration in the medium (µM), K_m is the Michaelis–Menten constant (µM), V_{max} is the maximum uptake rate (pmol/min/mg of protein) and P_{dif} is the nonspecific uptake clearance (µl/min/mg of protein). Fitting was performed by the nonlinear least-squares method using MULTI program [19] with the Damping Gauss Newton Method algorithm.

Results

Cellular localization of human OATP1B1

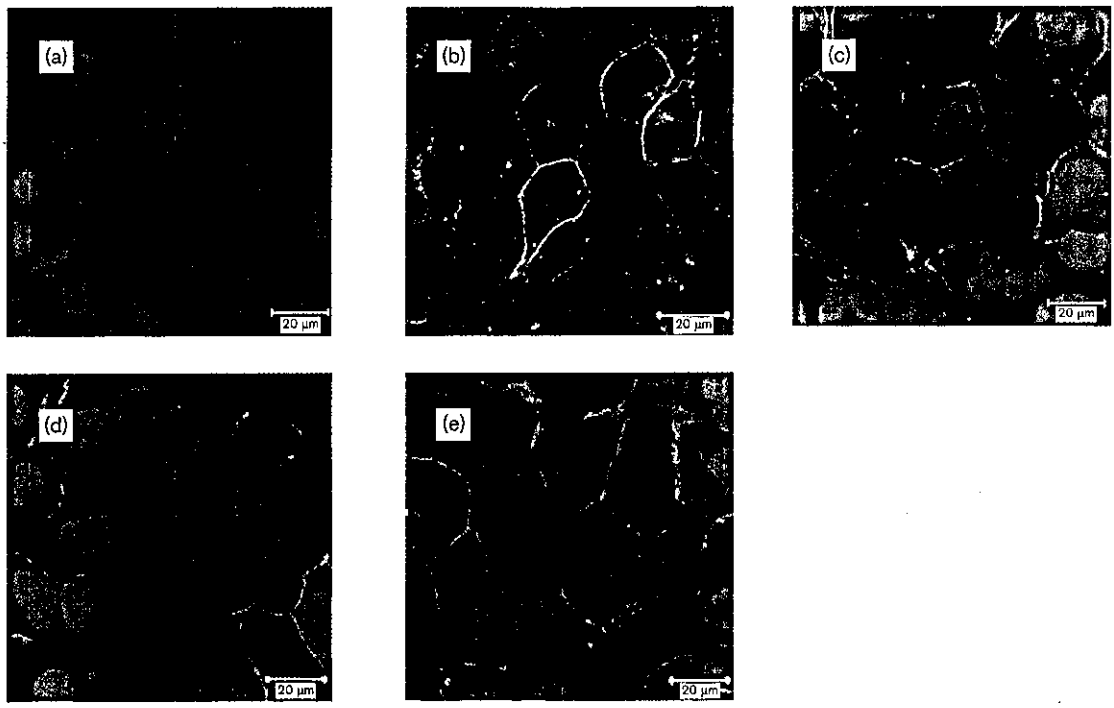
The localization of OATP1B1 SNP variants was investigated by immunocytochemical staining (Fig. 1). It has been previously reported that the localization of OATP1B1*1a and OATP1B1*1b is limited to the cell surface in transiently transfected HEK293 cells [14]. It was found that OATP1B1*1a and OATP1B1*1b are localized on the cell surface in HEK293 cells (Fig. 1), which is consistent with the previous report [14]. Moreover, cellular surface localization was identified for OATP1B1*5 and OATP1B1*15. No OATP1B1 derived staining was observed in vector-transfected HEK293 cells (Fig. 1).

The cellular localization of SNP variants was also confirmed by the cell surface biotinylation method (Fig. 2). The ratio of the biotinylated OATP1B1 in the cell surface fraction to that in the whole cell lysate was analyzed by densitometric analysis. This ratio of OATP1B1*1b, OATP1B1*5 and OATP1B1*15 was almost the same as that of OATP1B1*1a, and they were 64, 98 and 103% of OATP1B1*1a, respectively (Fig. 2). The efficiency of fractionation of cell surface protein was confirmed by an immunodetectable intracellular protein (calnexin).

Western blotting

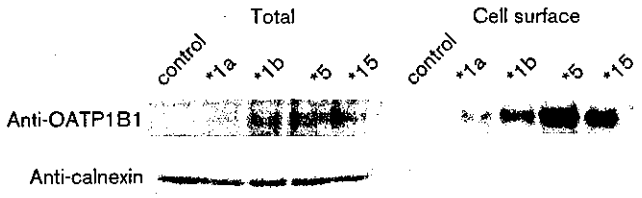
Western blotting was performed using crude membrane from HEK293 transfectants, and the protein expression level of each OATP1B1 SNP variant was estimated by quantifying the OATP1B1 band densities (Fig. 3). Although the expression level of each SNP variant differed, the molecular mass was approximately 80 kDa (Fig. 3). OATP1B1*1b and OATP1B1*5 showed a

Fig. 1



Immunolocalization of OATP1B1 allelic variants in HEK293 cells. OATP1B1 (green fluorescence) and nuclei (red fluorescence) were stained using the polyclonal antibody for OATP1B1 and propidium iodide, respectively. (a–e) represent the staining of vector-transfected, OATP1B1*1a-, OATP1B1*1b-, OATP1B1*5- and OATP1B1*15-expressing HEK293 cells, respectively. Bars = 20 μm.

Fig. 2



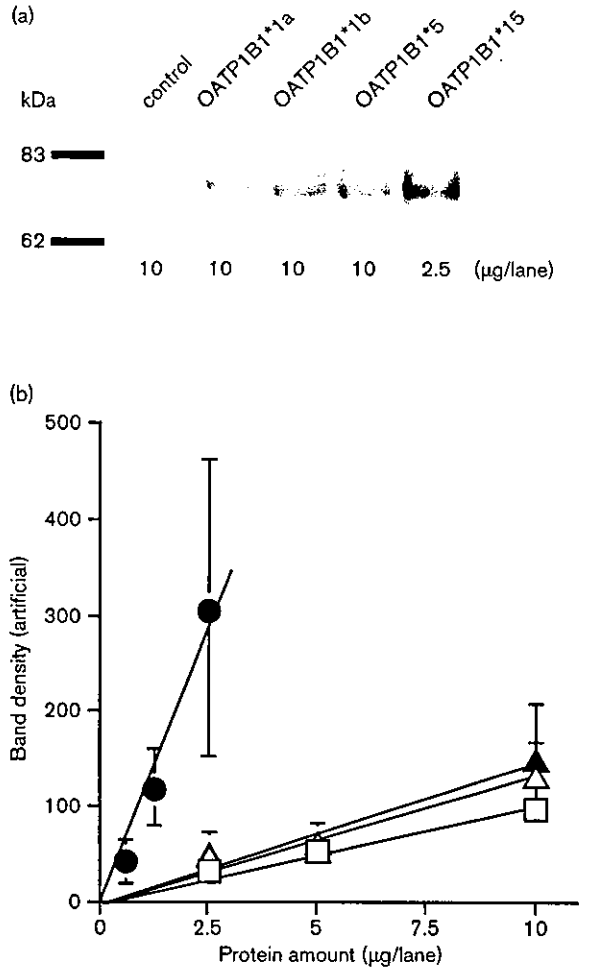
Cell surface biotinylation of OATP1B1 allelic variants. Total cell lysate protein containing both cell surface and intracellular protein (15 μl of solution) (left panel) and biotinylated protein (2 μl of solution) (right panel) was subjected to SDS–polyacylamide gel electrophoresis (PAGE) (7%). The membrane was incubated with anti-OATP1B1 polyclonal antibody (top panel). After washing, the membrane was incubated with anti-calnexin antibody (bottom panels). The band density against OATP1B1 was quantified in a luminescent image analyzer.

similar expression level to that of OATP1B1*1a (130% and 140%, respectively). In contrast, the expression level of OATP1B1*15 was approximately 11-fold higher than that of OATP1B1*1a. These values were used to normalize the transport activities of each OATP1B1 variant.

Uptake experiments

The time-profiles for the uptake of [^{3}H] $E_2$17βG and [^{3}H] E_1S by HEK293 cells stably expressing OATP1B1s are shown in Fig. 4. Significantly greater uptake of these compounds was observed in the cDNA-transfected cells compared with vector-transfectant (Fig. 4). Moreover, kinetic parameters were analyzed using [^{3}H] $E_2$17βG as a substrate (Fig. 5). The data for the saturation studies (Fig. 5) are given after correcting the uptake into vector-transfected cells. However, even after subtracting the transport into the vector-transfected cells, it was necessary for us to assume the presence of P_{dif} for the analysis, presumably due to the experimental deviations in the uptake between the vector-transfected and OATP1B1 cDNA-transfected cells. The K_m values of OATP1B1*1a, OATP1B1*1b, OATP1B1*5, and OATP1B1*15 were comparable, and they were 4.26 ± 1.66, 3.96 ± 1.29, 5.90 ± 2.00 and 4.07 ± 2.48 μM, respectively (Fig. 5). The apparent V_{max} values of these variants were 35.7 ± 12.9, 49.3 ± 14.9, 46.6 ± 15.1 and 28.8 ± 16.1 pmol/min/mg cellular protein, respectively. In order to determine the V_{max} values for each OATP1B1 molecule, it is essential to consider the cell surface expression level of OATP1B1s. Table 1 shows these values obtained by dividing the V_{max} values per mg cellular

Fig. 3



Quantification of the expression level of OATP1B1 in HEK293 cells. Crude membrane obtained from HEK293 cells was separated by SDS–PAGE (7%). The applied amount of protein was 10 μg, 10 μg, 10 μg, 10 μg and 2.5 μg for OATP1B1*1a, OATP1B1*1b, OATP1B1*5 and OATP1B1*15, respectively. OATP1B1 proteins were detected using horseradish peroxidase-labeled anti-rabbit IgG after incubation with the polyclonal antibody for OATP1B1 (a). Quantification of the OATP1B1 expression level was performed in a luminescent image analyzer. In (b), each point and bar represents the mean ± SE of three independent experiments, □, OATP1B1*1a expressing cells; △, OATP1B1*1b expressing cells; ▲, OATP1B1*5 expressing cells; ●, OATP1B1*15 expressing cells.

protein by the OATP1B1 expression level estimated by Western blotting (Fig. 3). For OATP1B1*15, the intrinsic V_{max} value was reduced to 7.3% of OATP1B1*1a, whereas the V_{max} values for OATP1B1*5 and OATP1B1*1b were similar to OATP1B1*1a.

Transport properties of other OATP1B1*15 clones

In order to confirm the results that the transport function of OATP1B1*15 is reduced compared to the wild-type transporter, further analysis was performed by using two other OATP1B1*15 clones. The results of the Western blot analysis and uptake study using OATP1B1*1a and other OATP1B1*15 clones (OATP1B1*15′ and OATP1B1*15″) are shown in Fig. 6. The expression level of OATP1B1*15′ and OATP1B1*15″ clones were 75 and 180% that of OATP1B1 *1a. OATP1B1*15′- and OATP1B1*15″-mediated transport was reduced to 21 and 30% of OATP1B1*1a, respectively, after correction of their protein expression levels (Fig. 6).

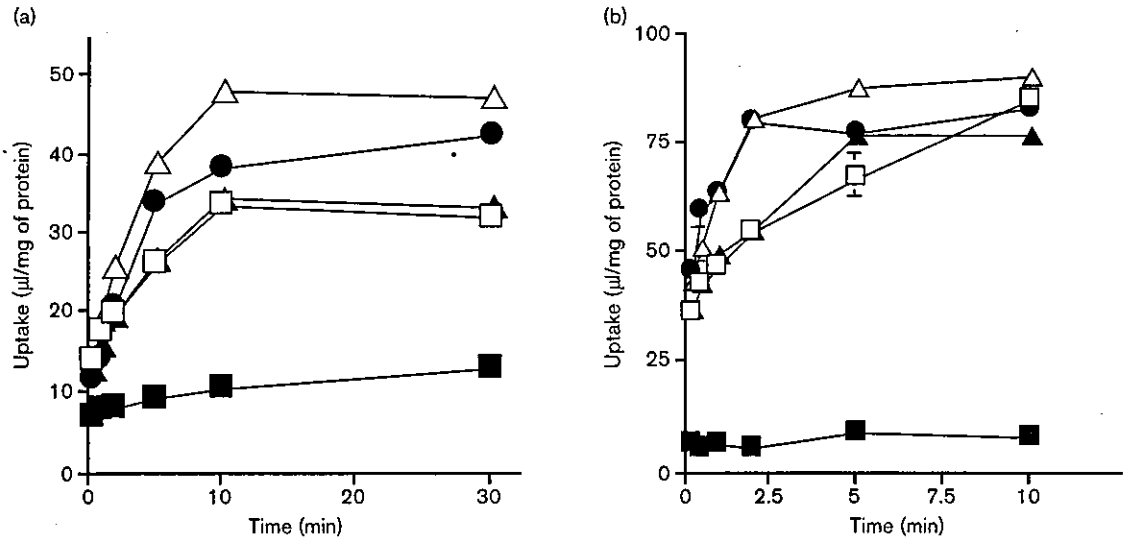
Discussion

In the present report, we focused on the function of Asn130Asp and Val174Ala SNP variants of human OATP1B1. The localization and transport activity were analyzed for OATP1B1*1a, OATP1B1*1b (Asn130Asp), OATP1B1*5 (Val174Ala) and OATP1B1*15 (Asn-130Asp and Val174Ala) using stably transfected HEK293 cells. The presence of Asn130Asp and Val-174Ala variations in the OATP1B1 gene has been reported in several ethnic groups. Among the SNP variations, Asn130Asp is observed at the highest frequency in European-Americans, African-Americans and Japanese and allelic frequencies of 30%, 74% and 60∼63%, respectively [13–15]. Although Val174Ala commonly appeared in European-Americans and Japanese with an allele frequency of 14% and 11∼16%, respectively, the frequency in African-Americans is relatively low (2%) [13]. Interestingly, the allelic frequency of OATP1B1*5, which has only the Val174Ala variation, is relatively low in Japanese (0∼0.7%), which is in contrast to the high frequency of OATP1B1*5 in European-Americans (14.0%) [13–15]. The Japanese subjects with Val174Ala also have Asn130Asp, and therefore, the presence of an interethnic difference in haplotype formation has been demonstrated between European-Americans and Japanese [13–15].

We recently reported that the non-renal clearance of pravastatin, an OATP1B1 substrate, in the Japanese subjects with the OATP1B1*15/*1b allele was reduced to 55% that in OATP1B1*1b/*1b subjects [15]. In addition, the same value in an OATP1B1*15/*15 subject was reduced to 14% of the control subjects [15]. It is possible that the reduction in OATP1B1 function by SNP variations is responsible for this functional alteration. The reduction in the function of membrane transporters may be accounted for by considering several factors: such as (1) the reduction in the intrinsic activity per transporter molecule, due to the reduced affinity for substrates and/or the reduction in the translocation ability; (2) altered protein expression due to the altered stability of mRNA and/or protein; and (3) impairment of the membrane sorting.

In the present study, we have characterized the SNP variants of OATP1B1 from the standpoints described above. The localization of SNP variants was analyzed

Fig. 4



Time-profiles for the transport of [^{3}H] $E_2$17βG and [^{3}H] E_1S by OATP1B1 SNP variants. Time-profiles for the uptake of [^{3}H] $E_2$17βG (100 nM; a), and [^{3}H] E_1S (50 nM; b) were examined. Each point and vertical bar represents the mean ± SE of three independent determinations. Key: ■; vector-transfected cells, □; OATP1B1*1a expressing cells, △; OATP1B1*1b expressing cells, ▲; OATP1B1*5 expressing cells, ●; OATP1B1*15 expressing cells.

by immunocytochemical staining and cell surface biotinylation methods. As shown in Fig. 1, most of the SNP variant molecules were located on the plasma membrane. In addition, these results were confirmed by the biotinylation assay (Fig. 2). The fact that OATP1B1*1a, OATP1B1*1b and OATP1B1*5 are mostly located on the plasma membrane is consistent with the previous results reported in transiently transfected HEK293 cells. However, the band density of OATP1B1*15 was not much higher than that of OATP1B1*1a/1b in Fig. 2. At the present moment, we do not have any good reason for this discrepancy between Figs 2 and 3. In obtaining the results shown in Fig. 2, the applied amount was not normalized with respect to the amount of lysate protein due to the difficulties in measuring the protein concentrations in the lysate solution containing SDS as a detergent. It is still possible that the applied amount differs between clones, although experiments were performed under the same conditions for all OATP1B1 variants. Since the degree of expression of OATP1B1 proteins is quite important in the interpretation of the experimental results, we also examined the transport mediated by OATP1B1*15 by using two other clones (Fig. 6) as discussed later in detail.

To compare the intrinsic transport activity of SNP variants of OATP1B1, the uptake of typical substrates was examined. The K_m values for $E_2$17βG were similar, indicating that the SNPs do not affect the affinity for $E_2$17βG (Fig. 5). The V_{max} values for $E_2$17βG were normalized by the protein expression level estimated by Western blotting. Although the V_{max} values defined for the amount of cellular protein were almost identical for OATP1B1*1a, OATP1B1*1b, OATP1B1*5 and OATP1B1*15, the intrinsic V_{max} for OATP1B1*15, defined for the expressed OATP1B1 protein level, was reduced to 7.3% of OATP1B1*1a (Table 1). Since the non-renal clearance of pravastatin, which is largely accounted for by biliary excretion clearance, in Japanese subjects with the OATP1B1*15 allele was reduced to 13% of that in OATP1B1*1a subjects [15], the degree of reduction in the transport activity is similar to the present in-vitro results. In contrast to the reduction in the transport activity of OATP1B1*15, no significant difference was observed in the kinetic parameters between OATP1B1*1a, OATP1B1*1b and OATP1B1*5 (Fig. 5).

The results that the transport function of OATP1B1*1a and *1b is similar for $E_2$17βG is consistent with the previous report by Michalski *et al.* [12]. In contrast, they found that the transport function of OATP1B1*1b is higher and lower for bromosulfophthalein and taurocholic acid, respectively, compared to OATP1B1*1a [12]. Since it is possible that the effect of SNPs on the transport activity is substrate-specific, it is important for us to examine the transport of pravastatin by OATP1B1 variants. Although we examined the uptake of pravastatin, we could not detect any significant OATP1B1-