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「工業動物細胞」のゲノム解析
10 g/l に向けたチャレンジ

動物細胞の培養は微生物と比べてその歴史が浅く，1907年に R. G. Harrison によって本格的に始められてから約100年弱の歴史しかないが，今ではインターフェロンやエリスロポエチンをはじめとして，近年活発になってきている抗体医薬[1]に至るまで多くの医薬品の生産手段として多用されている．さらに，新しい利用法として細胞自身を治療や評価に用いる手法（再生医療，バイオ人工臓器，細胞療法，毒性評価など）にも広がってきている．

さて，「工業動物細胞」とは筆者の造語であるが，実際にタンパク質医薬品の工業的生産にはどのような細胞が多用されているのであろうか．2006年にアメリカとEUにおいて実際に上市されているバイオ医薬品[2] 107品目（抗体医薬29品目を除く）について調べてみると，大腸菌が42%，チャイニーズハムスター卵巣（CHO）細胞が29%，*Saccharomyces serevisiae* が21%と，この3つの宿主細胞で実に90%以上のバイオ医薬品が生産されていることがわかる．「工業動物細胞」すなわちCHO細胞と置き換えても過言ではないであろう．

動物細胞培養を研究の手段として用いる出発点は，細胞を生体外に取り出し，これを培養する技術の開発にある．CHO細胞は1958年にPuckらによってチャイニーズハムスター卵巣組織から樹立され，Kaoらにより1968年に分離された亜種CHO-K1細胞株がATCCに登録されている．現在，産業応用されているCHO細胞株はこのCHO-K1由来細胞株である．CHO細胞がタンパク質医薬品生産に多用されている理由としては，①GMP (Good Manufacturing Practice) での生産実績がある，②無血清培地に馴化（浮遊培養）が容易であり，産業用培地が数多く開発されている，③遺伝子増幅（後述）などの高発現系が利用可能であり，組換え細胞が構築しやすい，④高密度流加培養* や灌流培養の実績とノウハウがある，などが挙げられる．現在では，CHO細胞を用いた抗体生産では最大濃度4 g/l の流加培養が実現可能となり，15,000 l 規模の培養槽での生産プラントが稼働している[3,4]．また，ゲノム解析やプロテオーム，メタボローム解析の発達をうけて，近年では，大腸菌や酵母と同様にCHO細胞を用いた物質生産においても，より高生産を目指したCHO細胞の詳細な解明が行なわれるようになってきている．

ミネソタ大学のHuらのグループは，シンガポールA STAR Instituteの研究グループとともに遺伝子増幅前のCHO親株およびインターフェロンγ産生CHO細胞株からのcDNAライブラリー（EST）の作製を試み，4,608個のESTを構築し，2,602個の特異的配列を得ている[5]．得られた配列は主としてマウスに最も相同性が高く，ついでヒトやラットに対しても高い相同性を示している．Huらのグループはこれを用いてcDNAマイクロアレイを構築し，様々な培養条件や高生産株における発現遺伝子や，その発現量の違いについて解析し，高生産培養実現のメカニズムの解明を図ろうとしている．また，得られた成果についてはCHOコンソーシアムを結成し米国内企業に対して公開，利用を呼びかけている．

*流加培養：バッチ式の培養において，培養液を抜き出すことなく培地を連続あるいは間欠的に供給する培養法

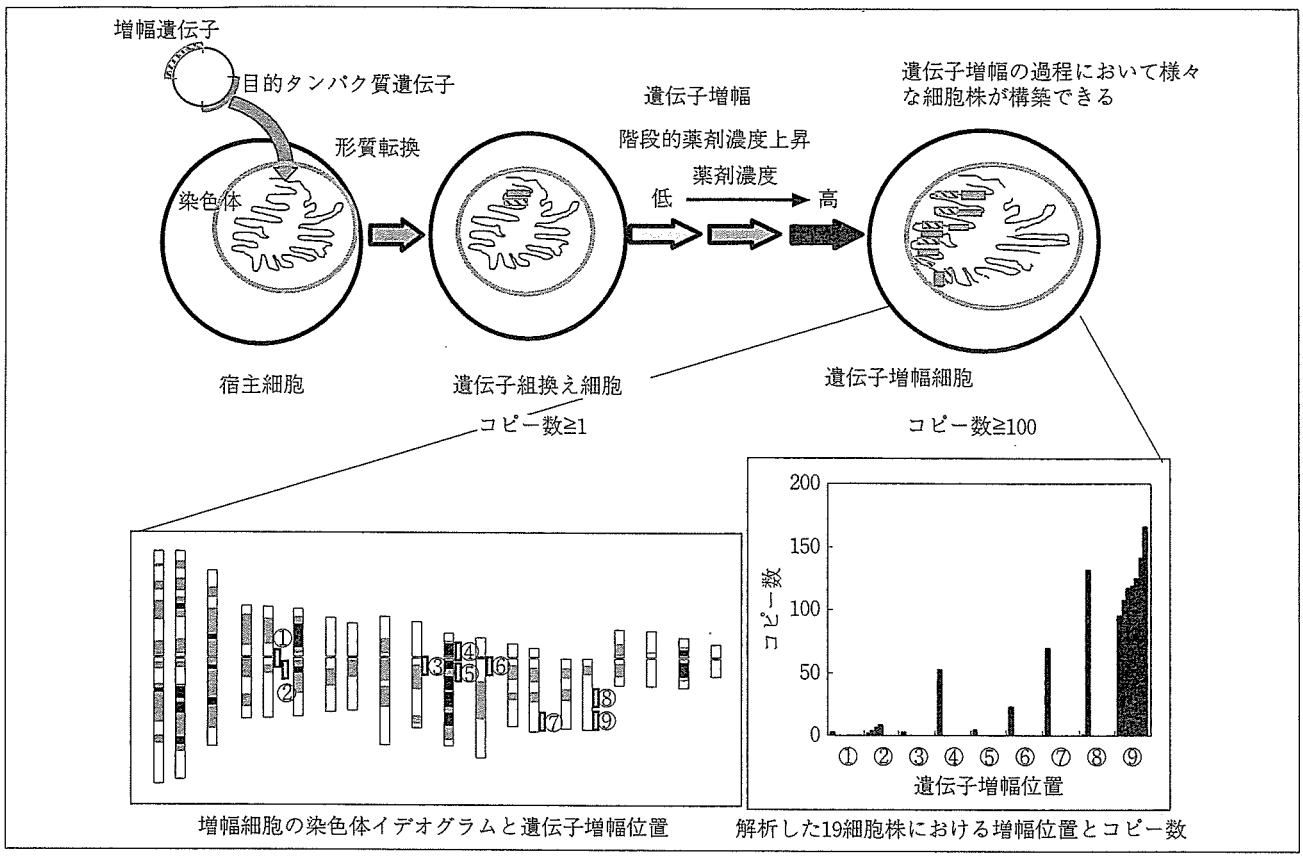

図1■遺伝子増幅による遺伝子増幅細胞株構築と染色体上の遺伝子増幅位置

一方，これとは別にCHO細胞の染色体を解明して細胞株の高度化を図ろうという研究も進められている．一般に，CHO細胞において高発現細胞株を構築する際には遺伝子増幅を利用している．本手法では，まず，コピー数増大をひき起こすことのできる増幅遺伝子（ジヒドロ葉酸合成酵素や薬剤耐性酵素などが知られている）と目的タンパク質の遺伝子を同時に細胞に導入する．次に，増幅遺伝子の発現する酵素に対する阻害剤を含む培地を用いて，段階的に阻害剤濃度を上昇させた選択を行なうことにより，染色体の一部を巻き込みながら，導入した遺伝子が増幅され，目的遺伝子と増幅遺伝子が多コピー組み込まれた細胞が構築できる（図1）．構築された細胞は生産性や増殖性が様々に変化しており，その中から目的のタンパク質生産に適した細胞が得られる．遺伝子増幅の宿主として汎用されているCHO DG44細胞では，20本の染色体数をもつ細胞が最も多く観察されること，正常の染色体以外の染色体が観察されること，そしてCHOゲノム自身の安定性と組み込んだタンパク質遺伝子発現の安定性との間に直接的な関係は見られないことをDerouaziらは報告している[6]．また，Kimらは染色体上の遺伝子増幅領域と長期間選択圧のない培地で培養した際の関係について検討し，$(TTAGGG)_n$配列が増幅遺伝子配列の近傍に観察され，増幅遺伝子のゲノム上での安定に関与しているのではないかと報告している[7]．

筆者らは，CHO DG44細胞–ジヒドロ葉酸還元酵素遺伝子増幅系において，様々な遺伝子増幅株構築条件における増幅遺伝子の増幅位置と構築された細胞株の安定性について検討し，コピー数および安定性の異なる細胞では染色体上の増幅位置が異なることを示した[8]．さらに筆者らは，この得られた細胞株のFISH画像に基づいて画像解析を行ない，CHO細胞の標準的な核型画像パターン（イデオグラム）を作成した．このイデオグラムと構築した増幅細胞の染色体上の増幅位置を重ね合わせることにより，ある特定の染色体位置が高コピー数に関係していることを見いだしている（図1）．このような染

色体上に存在する高発現に関係する配列は Hot Spot と呼ばれているが，その配列はほとんど明らかになっていない．そこで筆者らは現在，得られているコピー数の高い CHO 細胞株から遺伝子増幅に関連する配列情報を得るために，慶応大学の協力のもと，CHO 細胞の全ゲノムを含む BAC ライブラリーを構築し増幅領域の解明を進めている[9]．チャイニーズハムスター（*Cricetulus griseus*）のゲノムサイズはゲノム計画が存在しないため正確にはわからないが，ヒトと同程度か，それ以上の 3,000～4,000 Mb と推定されている．現在，ゲノムをすべてカバーする 12 万個からなる BAC ライブラリーを構築し，増幅領域をスクリーニングしている．今後，構築した BAC を用いた BAC-FISH による染色体分類マーカーの開発や，増幅領域の配列情報を解析することによる遺伝子増幅メカニズムの解明，さらには染色体配列を用いた Hot Spot に特異的に組み込まれるベクターの開発が可能になるであろう．

CHO 細胞の最大抗体比生産速度は，筆者の知る限り，現在最適な流加培養を行なうことにより 50～80 pg/cell/day 程度（終濃度 6 g/*l*, 培養 16 日）にまで上昇している[10]．さらなる細胞濃度の高密度化や細胞増殖速度の大幅な改善は難しいため，現在の 1.5～2 倍程度の比生産速度をもった細胞株を構築できれば，10 g/*l* が実現可能になると考えられる．そのためには，これまでの経験的手法と徹底的なスクリーニングを用いた高発現細胞株の取得技術や培地条件の検討のみならず，既存細胞株のゲノムやプロテオーム，メタボローム解析に基づいた工業動物細胞としての最適な改造を行ない，生産性向上を目指すアプローチが必要となるであろう．

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抗血管新生作用を有するトコトリエノールの高生産イネの作出

イネ産業資源の多面的有効活用に向けて

筆者らは最近，コメ糠を原料にしたコメ油製造時の副産物であり，コメの特徴成分ではあるものの十分活用しきれていなかったトコトリエノール（T3）の生理作用を調べ，コメ T3 が強い抗血管新生作用を有することを見いだした[1~4]．我が国のような高齢社会では，糖尿病性網膜症，リュウマチ性関節炎，癌，動脈硬化などの血管新生病が増加しており，これへの対応が社会から望まれている．コメ T3 はその活性の強さから，血管新生病の予防に十分活用できると考えられる．近年，イネの全塩基配列が解読され，イネ遺伝子の発現制御の機構が解明されつつある[5,6]．筆者らは，イネの遺伝子情報を利用して，T3 を高生産できる新品種の作出技術の開発に取り組んできている．血管新生病の予防と治療に，T3 高生産イネから得られるコメ糠を新機能食材として活用しようとするのが筆者らのねらいである[7]．

T3 はビタミン E 同族体であり，高等植物におけるその生合成経路，またそれに関わる酵素の遺伝子研究も進んでいる．T3 は植物細胞の主に葉緑体でつくられる．T3 の前駆物質は，2-メチル-6-ゲラニルゲラニル-1,4-ベンゾキノン〔MGGBQ，ホモゲンチジン酸（HGA）とゲラニルゲラニル 2 リン酸（GGDP）の縮合物であり，ホモゲンチジン酸ゲラニルゲラニルトランスフェラーゼ（HGGT）の働きで生じる〕である（図 1）．大部分の MGGBQ はメチル化されて 2,3-ジメチル-5-ゲラニルゲラニル-1,4-ベンゾキノン（DMGGBQ）となり，トコフェロールサイクラーゼ（TC）の作用で環化されγ-T3 にな

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Minireview

ABCA7, a molecule with unknown function

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Abstract Many ABC proteins are functional in cellular lipid transport in various different manners. ABCA7 is a full-size ABC transporter, the physiological function of which is unknown to date. This is a protein that shows the highest homology known to ABCA1, an essential molecule for producing of plasma high-density lipoprotein (HDL), and in fact it mimics ABCA1 to mediate the production of HDL from cellular lipid when transfected in vitro. It is therefore rational to assume that ABCA7 plays a relevant role in regulating of lipid metabolism. However, the ABCA7 expression profile is distinct from that of ABCA1, with respect to tissue-specific distribution and response to some reagents, presumably because of different transcriptional and posttranscriptional regulation. Potential roles and functions of ABCA7 in lipid homeostasis are discussed, especially in relation to HDL metabolism, based on available publications.

Keywords: ABCA7; High-density lipoprotein; Apolipoprotein A-I

1. Introduction

ABCA7 is one of the ABC family proteins that is a so-called full-size ABC transporter consisting of two sets of the multiple membrane-spanning domains plus the Walker motifs for the ATP interaction [1]. ABCA7 is a protein having the highest homology known to ABCA1, one of the essential proteins for cholesterol homeostasis. Mammalian ABCA1 plays an important role in cellular and body cholesterol homeostasis, by mediating production of plasma high-density lipoprotein (HDL) with helical apolipoprotein and cellular lipid and thereby regulating cellular cholesterol release [2,3]. Therefore, it is assumed that ABCA7 may also function in lipid or cholesterol metabolism. When ABCA7 was transfected in vitro, it in fact mediates production of HDL upon the interaction of the cells with helical amphiphilic surface-active proteins such as apolipoprotein (apo) A-I, apoA-II and serum amyloid A protein, except that relative release of cholesterol to phospholipid is much lower than the ABCA1-mediated reaction [4–9, Abe-Dohmae et al. manuscript submitted]. However, deficiency of ABCA7 may not markedly alter the plasma HDL level or other lipid/cholesterol metabolism [8] and the tissue-specific expression profile is substantially different from that of ABCA1 [10–12]. Thus, the physiological function of ABCA7 is still essentially unknown. In this article, we intend to review the up-to-date information on ABCA7 and discuss potential functions of this protein based on the available published results.

2. Structure of ABCA7

Human [10], mouse [11], and rat [12] ABCA7 cDNAs have been cloned from macrophages, thymus, and platelets, respectively, and the clones exhibited high homology to other ABC transporters. The cDNAs of ABCA7 encode proteins of 2146 (human) [10], 2159 (mouse) [11], and 2170 (rat) [12] amino acids, respectively. Human ABCA7 protein shows the highest homology known to human ABCA1 (54%) and human ABCA4 (49%) [10]. Interspecies identity of protein sequences of ABCA7 is 79% between human and mouse, less than that of ABCA1 (95%) and ABCA4 (88%) genes [11]. ABCA7 proteins of all the three species contain two sets of ATP-binding cassettes including Walker A and B motifs, which are present in many ATP-utilizing proteins, and a Walker C signature motif, characteristic to the full-size ABC transporters [1]. Interestingly, a cDNA of human ABCA7 has been also cloned from thymus [13] as a gene encoding SS-N, an epitope of an autoantigen related to Sjögren's syndrome.

A splicing variant mRNA (type II mRNA) of human ABCA7 has been detected [5]. The new exon found between exon 5 and 6, which was designated as exon 5B, contained two terminal codons and another translation initiation codon. Thus type II mRNA produces ABCA7 protein containing 28 novel N-terminal amino acids instead of the 1–166 amino acid sequence in full length (type I) ABCA7. Tissue-specific alternative splicing was detected by RT-PCR. Among the tissues examined, expression of type II mRNA was higher than type I mRNA in spleen, thymus, lymph node, and trachea, while type I mRNA was equal to or more than type II mRNA in bone marrow and brain [5]. In this review, type I ABCA7 and type II ABCA7 are designated as ABCA7 and type II ABCA7, respectively.

In Western blotting analysis, protein bands of different molecular weight have been demonstrated in some tissues from mouse [4] and rat [12], suggesting alternative splicing and/or alternative posttranslational modifications.

3. Clinical relevance and gene disruption

No genetic defect was reported on the human ABCA7 gene. A high-density single nucleotide polymorphism (SNP) map of

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ABC transporters in Japanese has been constructed and identified 67 SNPs at the ABCA7 locus [14]. No association has been indicated between variations and pathological phenotypes to date.

Analysis of human ABCA7 gene structure revealed that the ABCA7 gene localizes in tandem with the minor histocompatibility antigen HA-1 on the same strand [15]. Studies on SNPs in the HA-1 gene demonstrated that the incidence of an allele (168 His) was lower in the patients with Sjögren's syndrome than controls [16]. In addition, SS-N is a part of the first extracellular domain of ABCA7 [13] and an antibody against this domain reacted to infiltrating plasma cells in salivary glands from the patients of this disease, but not in those from nonspecific sialoadenitis patients [17]. The pathophysiological significance of these observations is unclear to date.

A homozygous ABCA7 knockout mouse was found embryonic lethal [7], but one other group reported generation of ABCA7 null mice [8]. No remarkable phenotype was observed in these animals, except that white adipose tissue mass was about 50% less and the serum HDL cholesterol level was somewhat but significantly lower than that of the wild type control only in the female mice at the age of 10-weeks. No such change was detected in the male knockout mice [8]. No difference was shown for apoA-I and apoE protein levels in the HDL or serum free fatty acid profile [8]. Production of the homozygous mouse by intercrossing of the heterozygotes was at the expected rate, and its development, including feeding behavior and weight gain up to 10 weeks, was normal [8]. These results indicate that ABCA7 deficiency in human might be also asymptomatic and no patient may have been found even if present.

There are no data available for hormone-dependent events and fertility of the knockout mouse. Analysis of gonadal steroid hormone would be helpful to address these issues.

4. Expression pattern

4.1. Tissue distribution

The ABCA7 gene is widely expressed both in adult and embryonic tissues in human [5,10], mouse [11], and rat [12]. The tissue distribution profile of ABCA7 is different from that of ABCA1. While the latter is ubiquitously expressed and especially abundant in the liver [2], RNA dot blot analysis of human tissues demonstrated the highest expression of ABCA7 in the thymus and predominant expression in other immune and hematopoietic tissues (spleen, lymph node, peripheral leukocytes, and bone marrow) [10]. In addition to these tissues, Northern blot analysis [5] showed high-level mRNA expression in the brain. In mouse, a similar expression pattern was observed by Northern blotting showing the highest mRNA levels in myelolymphatic tissues [11]. mRNA was also detected in the brain, adrenal glands, and uterus [11]. In the brain, neuronal staining was demonstrated by in situ hybridization analysis [8].

Data are limited for the tissue distribution of ABCA7 protein in human, but the expression pattern of ABCA7 protein in mouse and rat is almost identical to the mRNA expression. In mouse, the ABCA7 protein level is high in the spleen, lung, adrenal glands, and brain, moderate in the peripheral macrophages and lymphocytes, and low in the liver [4]. High-level expression of ABCA7 protein was also detected in platelets [4], erythrocytes [4], and in the white adipose tissue [8]. ABCA7 protein was highly expressed in platelets in rat, and much lower in lymphocytes, erythrocytes, brain, and ovary [12]. Analysis of the ABCA7 expression in human peripheral blood cells gave controversial results. One article reported expression of ABCA7 in lymphocytes, granulocytes, and monocytes [10] but the other claimed the failure of detection of ABCA7 protein in peripheral blood cells in spite of the mRNA expression [5].

4.2. Subcellular localization

Little is known concerning the subcellular localization of endogenous ABCA7 protein. Immunofluorescence confocal microscopy with rabbit anti-mouse ABCA7 antibody detected no signal at the plasma membrane but in the intracellular space in peritoneal macrophages [7]. The same authors described positive staining of the brush border membrane in proximal tubules of the kidney [7], but the background in the control staining was significant [7] and expression levels of ABCA7 mRNA and protein were both reportedly low in the kidney [4,8,11,12].

ABCA7 protein resulting from transfected cDNA (wild type, GFP-tagged, and Flag-tagged) was localized predominantly in the plasma membrane but was also detected in the intracellular membranes [4–7,12]. When expressed in CHO cells, rat ABCA7 with no tag and that with GFP tag at the N-terminus were both detected mainly in the plasma membrane, while ABCA7 with GFP fusion to the C-terminus was localized in the perinuclear membrane as well being probed by the ABCA7 epitope and GFP fluorescence [12]. Cell surface biotinylation [4] and immunofluorescence microscopy analysis of non-permeabilized cells with the antibody against the first putative extracellular domain of human ABCA7 (amino acids 45–549) [5] confirmed the cell surface expression of the ABCA7 protein. The latter experiment also revealed that this domain is exposed to the outside of the cell indicating the same topological arrangement as ABCA1 [5,13,18]. Most of type II ABCA7 protein was found intracellularly localized when expressed in HEK 293 cells [5] indicating that the N-terminal domain is important for sorting ABCA7 protein to the plasma membrane.

5. Regulation

5.1. Transcriptional regulation

The predicted promoter regions of human and mouse ABCA7 conserved two modules containing putative transcription factor-binding sites. The sites are targets for either ubiquitous transcription factors or liver- or lymphoid-specific transcription factors [11]. No data are available on the contribution of these regions to the promoter activity.

ABCA7 is assumed to be a sterol-regulated gene, because mRNA and protein levels of human monocyte-derived macrophages were reportedly increased by adding acetyl-low-density lipoprotein to the medium and decreased by HDL3 [10]. The liver X receptor (LXR) and the retinoid X receptor (RXR) are major transcriptional regulators of the ABCA1 gene [2]. However, the ABCA7 mRNA level in mouse peritoneal macrophages was not affected by an LXR agonist with and without an RXR agonist [4]. The ABCA7 protein level of the

mouse bone marrow-derived macrophages was also unaffected by stimulation with free cholesterol and LXR/RXR agonists [8].

We have recently found that ABCA7 mRNA level is regulated by sterol responsive/regulatory element binding protein 1a and 2 in human and mouse fibroblasts (Iwamoto et al., unpublished data). These findings should provide a new clue to understand regulatory mechanism of gene expression and biological roles of ABCA7.

5.2. Posttranscriptional regulation

Nothing has been reported for posttranscriptional regulation of endogenous ABCA7. In the transfected cells, apoA-I and protease inhibitor *N*-acetyl-Leu-Leu-norleucinal (ALLN) increased the steady state level of human ABCA7-GFP protein in HEK293 cells [6]. We have shown that apoA-I and ALLN upregulate cellular ABCA1 protein level through inhibiting a thiol protease, most likely calpain [19,20]. Therefore, there might be a similar regulatory mechanism for ABCA7 protein metabolism. Phorbol 12-myristate 13-acetate (PMA) treatment of the same cells resulted in a slight increase of the ABCA7 protein but apoA-I-mediated cholesterol and phospholipid release was significantly inhibited. In contrast, in the ABCA1-expressing cells, both ABCA1 protein level and apoA-I-mediated lipid release were significantly upregulated by PMA. Gö6976, an inhibitor of Ca^{2+}-dependent protein kinase C isoform(s), reversed all these effects of PMA. Thus, protein kinase C is likely involved in posttranscriptional regulation of both ABC proteins, and it seems to modulate the specific activity of ABCA7 for release of cellular lipid and generation of HDL in vitro [6].

6. Functions

6.1. HDL generation

The physiological function of ABCA7 is unknown. Because of the structural homology to ABCA1, and because of the fact that many ABC transporters act as lipid transporters [13,18,21], the study on the function of ABCA7 has been focused on cellular lipid metabolism. ABCA1 was identified as a causative gene for familial HDL deficiency (Tangier disease) and other genetic HDL deficiencies [22–26] resulting in the lack of the apolipoprotein-mediated HDL generation from the cellular lipid [27,28]. Thus ABCA7 was investigated for the analogy to this type of reaction in the transfected cells [4–9] (Table 1).

All the results agree that ABCA7 supports apolipoprotein-mediated phospholipid release and that phospholipids are the primary substrate for this ABCA7-mediated reaction. ABCA7 expressed in several cell lines promoted phospholipid release by lipid-free apolipoproteins such as apoA-I and apoA-II in a time- and dose-dependent manner, similarly to ABCA1. As was reported in ABCA1 [29], HEK293 cells expressing mouse ABCA7 showed increased specific binding of apoA-I, and covalent cross-linking studies revealed complex formation of ABCA7 and apoA-I [4]. These data suggested that apoA-I binds ABCA7 directly, or localizes very close to ABCA7 on the cell surface. After normalization of cell surface protein expression with Flag-tag, mouse ABCA7 was shown to have the same "phospholipid transporter" activity [7] as ABCA1, or 10–50% of the ABCA1 activity [8].

ABCA7 mediates cholesterol release much less than phospholipid, though we claim it is low but positive by using human ABCA7 without or with GFP-tag expressed in several types of cell lines even in the absence of ABCA1 [5,6,9] and others report negative [4,7] or no [8] data (Table 1). It is noteworthy that type II ABCA7 protein, very little is localized in plasma membrane, mediates neither cholesterol nor phospholipid release [5].

According to our data, the released cholesterol/phospholipid ratio from ABCA1-expressing cells is much higher than that from ABCA7-expressing cells [6,9]. We have developed a HEK 293 cell system with ecdysone-inducible human ABCA1 and ABCA7. While the cholesterol/phospholipid ratio in the apolipoprotein-released lipid increased along with the increase

Table 1
ApoA-I-mediated lipid release by ABCA7

Cell	cDNA	Tag	Expression[d]	Additional treatment	Ch release[e]	PL release[e]	Reference
HEK293	Human	–	T	–	+	+	[6]
		–	T	–	–	+	[7]
		GFP	T	–	+	+	[5]
		GFP	T	–	+	+	[6]
		GFP	S	–	+	+	[6]
HEK293 ECR[a]	Human	GFP	I	–	+	+	[9]
HEK293	Human type II	GFP	T	–	–	–	[5]
L929[b]	Human	GFP	SM	–	+	+	[6]
HEK293	Mouse	–	T	–	–	+	[4]
		–	T	–	–	+	[7]
		–	T	–	ND	+	[8]
293-EBNA-T	Mouse	–	T	–	ND	+	[8]
HEK293	Mouse	Flag	T	–	ND	+	[4]
		Flag	T	–	ND	+	[8]
		–	T	Ch loading	–	ND	[4]
		–	T	SR-BI expression	–	ND	[4]
CHO[c]	Mouse	–	T	–	–	ND	[4]

[a]HEK293 cells expressing ecdysone receptor.
[b]Parent cells are positive for apoA-I-mediated phospholipid release but negative for cholesterol release.
[c]Parent cells are positive for apoA-I-mediated cholesterol and phospholipid release.
[d]I, S, SM, and T denote inducible, stable, mixture of stable clones, and transient, respectively.
[e]ND: not described.

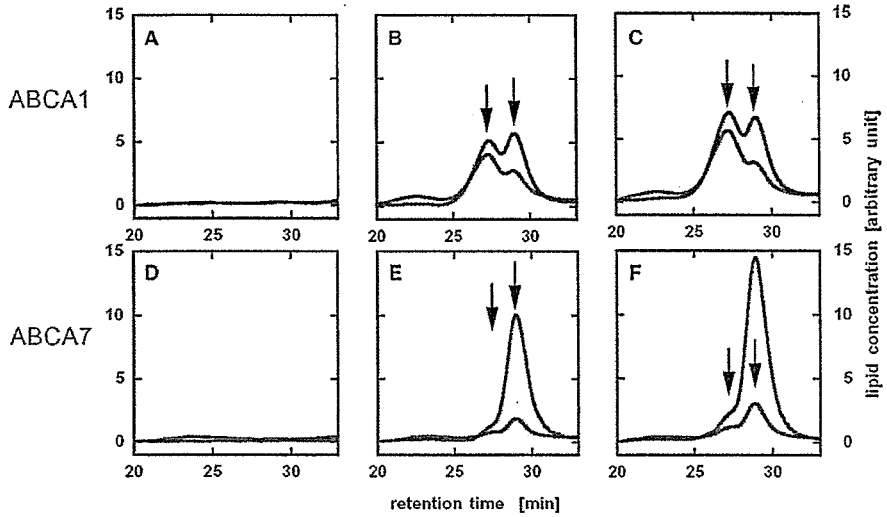

Fig. 1. Lipoprotein analysis of the HDL particles generated from the ABCA1- and ABCA7-induced cells (taken from Ref. [9]). Ecdysone receptor-expressing HEK293 cells with inducible human ABCA1-GFP (A–C) and inducible human ABCA7-GFP (D–F) were incubated without (A,D), with low dose (B,E), and with high dose (C,F) of ponasterone A for induction. After 24-h incubation with 10 μg/ml apoA-I, medium was collected for HPLC analysis. The concentration of cholesterol (red lines) and phospholipid (blue lines) in each fraction was measured.

of ABCA1 protein level by ecdysone, the ratio remained constant when the ABCA7 protein level was increased [9]. Analyses of size distribution and lipid composition of the generated HDL particles by a gel permeation HPLC system revealed that the ABCA1-mediated reaction produced two types of HDL particles, large particles with a high cholesterol/phospholipid ratio and small particles with a low cholesterol/phospholipid ratio (Fig. 1B). The ABCA7-mediated reaction generated mostly the small particles (Fig. 1E). Induction of the ABC transporters by ecdysone caused a dominant increase of the large particles in the ABCA1-expressing cells and only the small particles in the ABCA7-expressing cells (Fig. 1C and F). Despite all these in vitro findings on the ABCA7-mediated generation of HDL, it is clear that ABCA1, not ABCA7, is essential to maintain the plasma HDL concentration from the studies on Tangier disease patients and ABCA1 knockout mice [2]. In addition, apoA-I-mediated cellular cholesterol and phospholipid release were all unaffected in bone marrow-derived macrophages prepared from ABCA7 knockout mice [8], peritoneal macrophages from ABCA7 heterozygous mice [7], and normal peritoneal macrophages treated with ABCA7 siRNA [7]. Therefore, the ABCA7-mediated lipid release may not significantly contribute to a source of plasma HDL. However, it may still play an important role in cellular cholesterol homeostasis in certain particular tissues including macrophages.

6.2. Others

ABCA7 is also expressed in several cells other than the reticuloendothelial system. Platelets express high-levels of ABCA7 at least in mouse and rat [4,12]. As platelets secrete various lipid mediators including sphingosine 1-phosphate and lysophosphatidic acid, possible functional integration of ABCA7 in the secretion mechanism of the mediators was suggested [12].

HaCaT cells, a spontaneously immortalized human keratinocyte cell line cells, and primary cultured normal human epidermal keratinocytes (NHEK cells) can be differentiated by increased Ca^{2+} concentration in the medium. During this differentiation period, the cellular ceramide concentration increases. RT-PCR analysis of the cells found that ABCA7 mRNA increased 3 times from the undifferentiated to the differentiated stage of NHEK cells [30]. Expression of human ABCA7 in HeLa cells resulted in an increase of intracellular phospholipids compared to the mock-transfected cells (to 135%, 130%, and 115% in ceramide, phosphatidylserine, and phosphatidylcholine, respectively) and an increase of cell surface ceramide expression [30]. From these data, a potential role of ABCA7 in epidermal lipid reorganization was proposed.

The ABCA7 protein level is high in mouse white adipose tissue and is upregulated during differentiation of the mouse preadipocyte cell line 3T3L1 to adipocytes, suggesting its potential role in lipid transport in these cells [8]. In the differentiated 3T3L1 cells, some ABCA7 protein is shown colocalized with perilipin in the lipid droplet and microsomal fractions, being different from ABCA1 [8].

7. Conclusions and perspectives

ABCA7 is a protein that has the highest homology to ABCA1. ABCA7 mediates apolipoprotein-derived generation of HDL similarly to ABCA1, suggesting that it may compensate the function of ABCA1 for release of cellular lipid in a certain condition(s). However, it is obvious that ABCA7 does not rescue the plasma HDL deficiency caused by the defect of ABCA1 function, in either man or mouse. In addition, differences between ABCA1 and ABCA7, especially in tissue distribution profile and transcriptional regulatory mechanism, implicate that ABCA7 may have a more specific role(s) than mimicking ABCA1. Studies on similarity and difference between ABCA1 and ABCA7 would provide us with more information to address the questions for understanding functions of these transporters.

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Serum amyloid A generates high density lipoprotein with cellular lipid in an ABCA1- or ABCA7-dependent manner

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Abstract **Serum amyloid A (SAA) is an amphiphilic helical protein that is found associated with plasma HDL in various pathological conditions, such as acute or chronic inflammation. Cellular lipid release and generation of HDL by this protein were investigated, in comparison with the reactions by apolipoprotein A-I (apoA-I) and several types of cells that appear with various specific profiles of cholesterol and phospholipid release. SAA mediated cellular lipid release from these cells with the same profile as apoA-I. Upregulation of cellular ABCA1 protein by liver X receptor/retinoid X receptor agonists resulted in an increase of cellular lipid release by apoA-I and SAA. SAA reacted with the HEK293-derived clones that stably express human ABCA1 (293/2c) or ABCA7 (293/6c) to generate cholesterol-containing HDL in a similar manner to apoA-I. Dibutyryl cyclic AMP and phorbol 12-myristate 13-acetate, which differentiate apoA-I-mediated cellular lipid release between 293/2c and 293/6c, also exhibited the same differential effects on the SAA-mediated reactions. No evidence was found for the ABCA1/ABCA7-independent lipid release by SAA. Characterization of physicochemical properties of the HDL revealed that SAA-generated HDL particles had higher density, larger diameter, and slower electrophoretic mobility than those generated by apoA-I. These results demonstrate that SAA generates cholesterol-containing HDL directly with cellular lipid and that the reaction is mediated by ABCA1 and ABCA7.**—Abe-Dohmae, S., K. H. Kato, Y. Kumon, W. Hu, H. Ishigami, N. Iwamoto, M. Okazaki, C-A. Wu, M. Tsujita, K. Ueda, and S. Yokoyama. **Serum amyloid A generates high density lipoprotein with cellular lipid in an ABCA1- or ABCA7-dependent manner.** *J. Lipid Res.* **2006.** 47: **1542–1550.**

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Serum amyloid A (SAA) is a protein family that comprises acute-phase and constitutive members, both of which are synthesized mainly in the liver and the former is in reaction to the inflammatory status (1). Although constitutive SAA (e.g., SAA4 in human) is identified only in human and mouse, acute-phase SAA (e.g., SAA1 and SAA2 in human) is found in all of the vertebrates investigated and is highly conserved across evolutionarily distinct species (1). Acute-phase SAA is a major acute-phase reactant, showing up to 1,000-fold increase in human plasma during inflammation (2). Increase of this protein is also found with chronic inflammatory diseases. Acute-phase SAA is experimentally induced in mice by intraperitoneal injection of lipopolysaccharide (LPS), a potent endotoxin in septic shock (3). SAA has a multisegment structure of amphiphilic helix that is very similar to helical apolipoprotein (4) and is found associated with HDL, especially HDL_3, in the circulation (5).

SAA may play an antipathogenic role in inflammatory processes as it binds to outer membrane protein A of Gram-negative bacteria (6). However, it could also be pro-inflammatory. LPS binds the scavenger receptor class B type I (SR-BI) and is internalized to stimulate cells to produce cytokines such as tumor necrosis factor-α (TNF-α), interleukin(IL)-1α, IL-1β, IL-6, and IL-8, IL-1ra, and soluble tumor necrosis factor receptor-II (TNFR-II) (7–9), and SAA itself may also contribute to the stimulation of cells via SR-BI to produce cytokines such as IL-1β, IL-8, IL-1ra, and soluble TNFR-II but not TNFα or IL-6, although

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Abbreviations: apoA-I, apolipoprotein A-I; dBcAMP, dibutyryl cyclic AMP; DF medium, 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium; D-PBS, Dulbecco's phosphate-buffered saline; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; SAA, serum amyloid A; SR-BI, scavenger receptor class B type I; TNF-α, tumor necrosis factor-α; TNFR-II, tumor necrosis factor receptor-II.

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to a lesser extent than LPS (7, 10). In addition, SAA is a precursor of amyloid A protein, the major component of amyloid fibrils in secondary amyloidosis (11, 12).

A more complicated argument is that SAA may modulate the effect of LPS through HDL metabolism. The effect of LPS is blocked by physiological ligands of SR-BI, HDL, apolipoprotein A-I (apoA-I), and amphiphilic α-helical peptides (9). As the HDL containing SAA competes with normal HDL for SR-BI binding (10, 13), an increase of such HDL may interfere with the effect of LPS. Infusion of HDL protected mice from the LPS-induced endotoxin shock status (14), which seems to be consistent with the view that HDL traps LPS from direct interaction with inflammatory cells in the interstitial space (15, 16). HDL particles containing SAA are different from regular apoA-I-containing HDLs in their physicochemical properties (3, 5), and they may result in different biological functions (1). Displacement of apoA-I from HDL by SAA causes an enhancement of HDL clearance and thereby a reduction of HDL (5, 17). A recent report, however, indicated that SAA, whether free or lipid-bound, could compete with HDL binding to SR-BI and interfere with cellular cholesteryl ester uptake from HDL (10, 13). Thus, reports of the roles of SAA in the pathogenesis of inflammation and HDL metabolism are controversial and unclear.

HDL plays a central role in cholesterol transport from extrahepatic peripheral cells to the liver as a major pathway for cholesterol catabolism. This pathway is also thought important to act against atherogenesis, as intracellularly accumulated cholesterol in the vascular wall can be removed by this mechanism. One of the rate-limiting steps of this pathway is the release of cellular cholesterol to HDL, and two independent mechanisms have been identified for this step (18): nonspecific cholesterol exchange between cell membrane and extracellular lipoproteins, and assembly of new HDL particles with cellular lipid and lipid-free helical apolipoproteins. The latter seems a major source of plasma HDL, as the cells from patients with familial HDL deficiency (Tangier disease) lack this reaction (19, 20). ABCA1 was identified as essential for HDL biogenesis (21–25). ABCA7 was also shown to mediate HDL biogenesis when expressed in HEK293 and L929 cells (26, 27).

Recently, SAA was reported to induce cellular lipid release both in ABCA1-dependent and -independent manners (28). Here, we have characterized the SAA-cell interaction in the context of HDL biogenesis using a series of cell lines. It was demonstrated that SAA is an analog of helical apolipoprotein and is fully capable of generating new HDL particles with cellular lipid in the presence of ABCA1 or ABCA7 in the cell membrane. HDLs generated with SAA appeared to have different physicochemical properties than those generated with apoA-I.

MATERIALS AND METHODS

Materials

ApoA-I was isolated from a human plasma HDL fraction (density, 1.09–1.21) and stored at −80°C until use as described previously (29). A stock solution (1 mg/ml) was prepared and stored at 4°C as described previously (30). SAA used in this study was recombinant human SAA corresponding to human SAA1α except for three amino acids (catalog number 300-13; PeproTech EC, London, UK), and a stock solution (1 mg/ml) was prepared according to the manufacturer's instructions and stored at 4°C. ApoA-I protein was undetectable in the SAA stock solution by Coomassie Brilliant Blue staining or Western blotting (data not shown). The sources of the other reagents were as follows: phorbol 12-myristate 13-acetate (PMA) and 9-*cis* retinoic acid were from Wako (Osaka, Japan); liver X receptor agonist TO901317 was from Sigma; dibutyryl cyclic AMP (dBcAMP) was from Seikagaku Corp. (Tokyo, Japan); and protein kinase C inhibitor Gö6976 was from Biomol Research Laboratories.

Cell culture

THP-1, HEK293, and L929 cells were obtained from the Health Science Research Resources Bank. HEK293 clones of 293/2c and 293/6c were established in our laboratory; they stably express human ABCA1-green fluorescent protein and ABCA7-green fluorescent protein, respectively (27). CHO-K1 cells, which produce cholesterol-containing HDL were from the American Type Culture Collection. The cells were maintained in medium supplemented with 10% (v/v) fetal calf serum under a humidified atmosphere of 5% CO_2 and 95% air at 37°C. RPMI 1640 medium was used for THP-1 cells, and a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF medium) was used for all other cells. For differentiation of THP-1 cells, cells were subcultured in six-well trays (Techno Plastic Products AG, Trasadingen, Switzerland, catalog number 92406) at a density of 3.0×10^6 cells/well in the presence of 320 nM PMA for 72 h (31).

Cellular lipid release assay

Cells were subcultured in six-well trays at the indicated density with 10% fetal calf serum-containing medium. After 48 h of incubation, the cells were washed once with Dulbecco's phosphate-buffered saline (D-PBS), except that buffer H [Hank's balanced salt solution containing 20 mM HEPES-KOH (pH 7.5) and 14 mM glucose] was used for HEK293, 293/2c, and 293/6c (27), and incubated in 1 ml/well DF medium containing 0.02% BSA (0.02% BSA/DF medium) and the compounds indicated. The PMA-treated THP-1 cells were further washed with D-PBS and incubated with apoA-I or SAA. The PMA-untreated THP-1 cells were directly subcultured in six-well trays at a density of 3.0×10^6 cells/well and cultured in 0.02% BSA/DF medium containing apoA-I or SAA. Lipid content in the medium and cells was determined after 24 h of incubation. Procedures for lipid extraction and enzymatic assays for cholesterol, cholesteryl ester, and choline-phospholipids were described previously (30).

Lecithin:cholesterol acyltransferase and lipase activities of the culture medium were determined using LCAT kit-S from Alfresa Pharma Corp. (catalog number 4987274862199; Tokyo, Japan) and an LIP Roche/Hitachi from Roche Diagnostics K.K. (catalog number 11821791 216; Tokyo, Japan), respectively.

Western blotting

A rabbit anti-ABCA1 antiserum (32, 33), a rat monoclonal anti-ABCA7 antibody, KM3095 (34), and a rat monoclonal anti-SAA antibody (Cell Sciences) were used to detect the indicated proteins.

Lipoprotein analysis

ABCA1- or ABCA7-transfected cells, 293/2c and 293/6c, were subcultured in 100 mm dishes at a density of 6.0×10^6 cells/dish,

cultured as described above, and stimulated with 5 ml/dish 0.02% BSA/DF medium containing 10 μg/ml SAA for 24 h. The conditioned medium was analyzed by sucrose density gradient ultracentrifugation as described previously (27, 35). It was also analyzed by an HPLC system with two tandem gel permeation columns (Lipopropak XL, 7.5 mm × 300 mm; Tosoh) at a flow rate of 700 μl/min and two parallel online enzymatic lipid detection systems (350 μl/min each) (36, 37) to evaluate the size distribution of the lipoprotein particles (38, 39) (Skylight Biotech, Inc., Akita, Japan). The system was calibrated by latex beads and high-molecular-weight standards for apparent spherical diameters of the subjects (36, 37).

For electron microscopy, the HDL fraction was isolated by ultracentrifugation from the conditioned medium at a density of 1.21 g/ml adjusted with NaBr or sucrose at 90,000 rpm in a Hitachi CS100 ultracentrifuge with an S100AT6 rotor (4.8×10^5 *g*) for 16 h (NaBr) or 48 h (sucrose) at 4°C. The top fraction (300 μl) of each tube (3 ml) was collected as HDL. The HDL fractions from eight tubes of the sucrose solution were pooled and further concentrated by centrifugation for another 48 h at 1.21 g/ml with sucrose. HDL samples were dialyzed against 10 mM ammonium bicarbonate, pH 7.9, at 4°C overnight. They were negatively stained with 0.5% uranium acetate and examined with a Hitachi 7100 electron microscope as described (40, 41). Long diameters (major axes) of the HDL particles were measured graphically using NIH Image 1.63 software.

To evaluate the electrophoretic mobility of lipoproteins, the conditioned medium was analyzed using an agarose gel electrophoresis system (Paragon System; Beckman) after concentration by an ultrafiltration filter (Amicon Ultra-15; Millipore).

Statistical analysis

Data were analyzed by one-way ANOVA followed by Scheffé's test. $P < 0.05$ was accepted as statistically significant.

RESULTS

Cellular lipid was released by SAA and apoA-I from THP-1 cells (**Fig. 1**). From the undifferentiated cells, cholinephospholipid was a major component, suggesting that the HDL generated was cholesterol-poor. When the cells were differentiated with PMA, the lipid release was increased but more prominently in cholesterol, indicating the generation of cholesterol-rich HDL. The lipid-release profile was also examined for CHO-K1, L929, and HEK293 cells (**Fig. 2**). These cell lines represent cells that release both phospholipid and cholesterol, phospholipid only, and no lipid, respectively, by apoA-I, as we reported in previous works (27, 42). The results with SAA were the same as those with apoA-I.

Transcriptional activation of the ABCA1 gene resulted in an increase of the SAA-mediated lipid release. A retinoid X receptor ligand, 9-*cis* retinoic acid, and a liver X receptor ligand, TO901317, increased ABCA1 synergistically in CHO-K1 cells (**Fig. 3A**). In these conditions, the lipid release mediated by apoA-I and by SAA both increased in parallel (Fig. 3B, C). That apoA-I increased ABCA1 is consistent with our previous reports that ABCA1 is stabilized against calpain by helical apolipoprotein (33, 43, 44), and SAA showed the same effect (Fig. 3A).

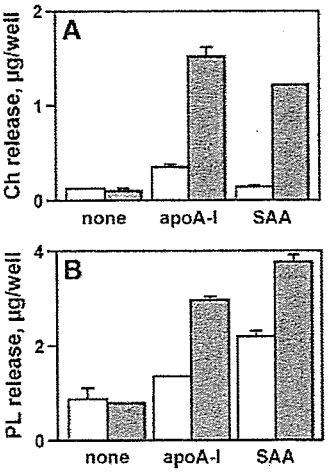

Fig. 1. Release of cholesterol (Ch; A) and choline-phospholipids (PL; B) from THP-1 cells. Undifferentiated (open columns) and differentiated (shaded columns) THP-1 cells were prepared as described in the text. Cells were washed once with Dulbecco's phosphate-buffered saline (D-PBS) and incubated with 0.02% BSA/DF medium (0.02% BSA and a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium) containing nothing (none), 10 μg/ml apolipoprotein A-I (apoA-I), or 10 μg/ml serum amyloid A (SAA) for 24 h. Cholesterol and choline-phospholipid in the medium were measured as described in the text. Data shown represent averages ± SD of duplicate measurements in one of three independent experiments yielding similar results. Error bars are not shown when they lie within the columns.

Figure 4 shows dose-dependent profiles of lipid release by apoA-I and SAA from cells to which ABCA1 or ABCA7 was transfected (293/2c or 293/6c, respectively). Although the ABCA1-mediated lipid release was nearly similar between apoA-I and SAA (Fig. 4A, C), the SAA-mediated lipid release was substantially less efficient with ABCA7 (Fig. 4B, D). dBcAMP and PMA had differential effects on the apoA-I-mediated cellular lipid release between ABCA1- and ABCA7-transfected cells, perhaps at posttranscriptional steps (27). These compounds showed very similar differential effects on SAA-mediated lipid release between the clones (**Fig. 5**). The SAA-mediated release of cholesterol and phospholipid from the ABCA1-transfected cells was increased by dBcAMP and PMA, and the effect of PMA was canceled by an inhibitor of protein kinase C, Gö6976 (Fig. 5A, C). In contrast, SAA-mediated lipid release from the ABCA7-transfected cells was not influenced by dBcAMP. PMA suppressed the ABCA7-dependent lipid release mediated by SAA, and Gö6976 canceled the effect (Fig. 5B, D). All of these results were consistent with our previous findings with apoA-I (27).

HDL generated by SAA was analyzed by density gradient ultracentrifugation (**Fig. 6A**). Cholesterol and phospholipid released from 293/2c were both recovered in the fractions with a density peak at ~1.10 g/ml, and those from 293/6c were recovered in the fractions with a density peak at 1.14 g/ml. SAA protein was detected in the lipid-containing fractions (data not shown). SAA used in this

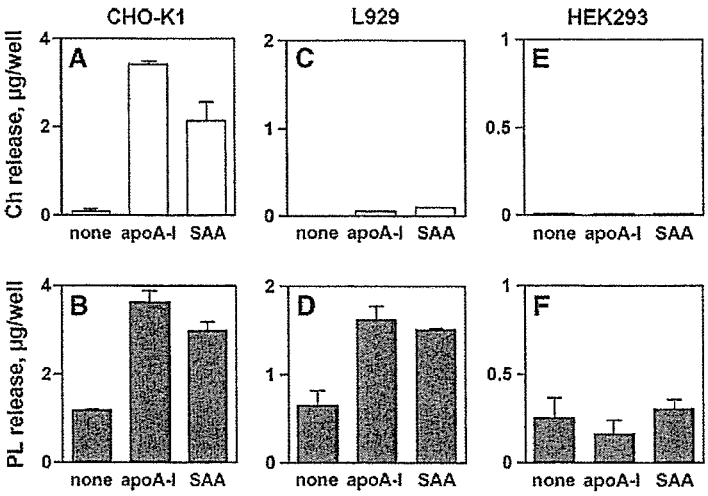

Fig. 2. Release of cholesterol (Ch; A, C, E) and choline-phospholipids (PL; B, D, F) from CHO-K1 (A, B), L929 (C, D), and HEK293 (E, F) cells. CHO-K1, L929, and HEK293 cells were subcultured in six-well trays at densities of 5.0×10^5, 5.0×10^5, and 1.0×10^6 cells/well, respectively. After 48 h of incubation, cells were washed once and treated as described for Fig. 1. Data represent averages ± SD of duplicate measurements in one of two independent experiments yielding similar results.

study was recombinant protein and had no detectable apoA-I (see Materials and Methods), so that SAA generated cholesterol-containing HDL from the cells in the presence of ABCA1 or ABCA7.

The size distribution of the HDL particles was analyzed by HPLC. The apoA-I-conditioned medium showed two peaks, consistent with our previous findings (39). Large cholesterol-rich particles are predominant in the HDL generated with the ABCA1-transfected 293/2c cells, whereas small cholesterol-poor particles are dominant with the ABCA7-transfected 293/6c cells (Fig. 6B). In contrast, the peaks found in the same analysis of the SAA-conditioned medium appeared large, single, broad, and rather symmetric (Fig. 6C).

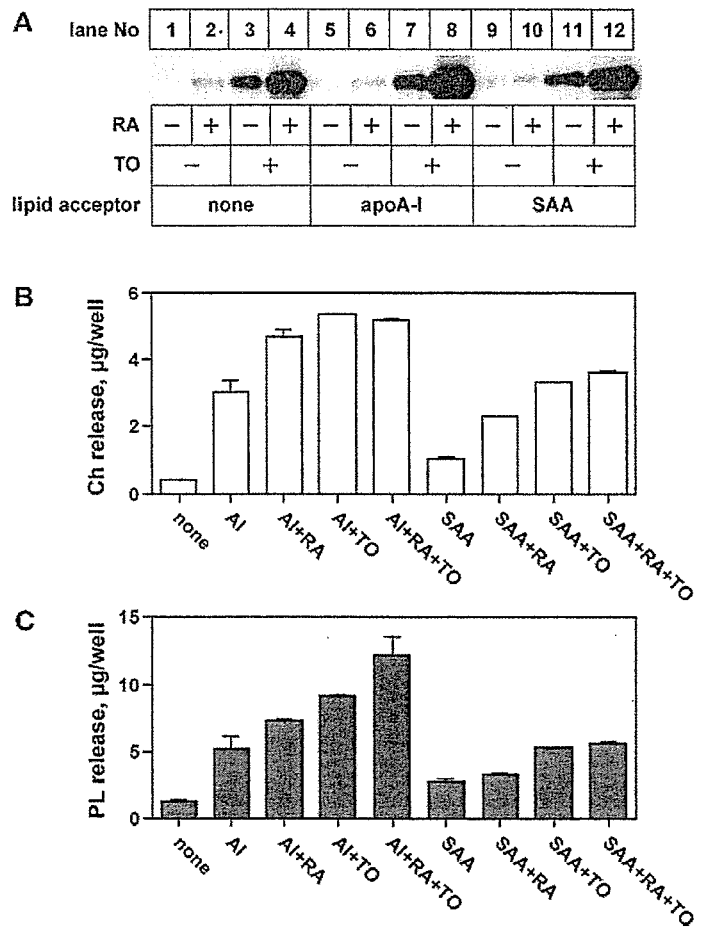

Fig. 3. Effects of 9-*cis* retinoic acid and TO901317 on cellular ABCA1 protein level and lipid release by apoA-I and SAA. CHO-K1 cells were subcultured in six-well trays at a density of 5.0×10^5 cells/well. After 48 h of incubation, cells were washed with D-PBS, and then 0.02% BSA/DF medium containing the compounds indicated was added. The final concentrations of 9-*cis* retinoic acid (RA), TO901317 (TO), apoA-I (AI), and SAA were 5 µg/ml, 10 µM, 10 µg/ml, and 10 µg/ml, respectively. Cells were cultured for another 24 h, and the cells and medium were collected for lipid measurement. The membrane fraction was prepared from the remaining cells for Western blot analysis of ABCA1. A: ABCA1 analysis by Western blotting. Membrane protein (100 µg/lane) was analyzed as described in the text. B, C: Release of cholesterol (Ch) and choline-phospholipid (PL), respectively, into the medium. Results shown are averages ± SD for two samples.

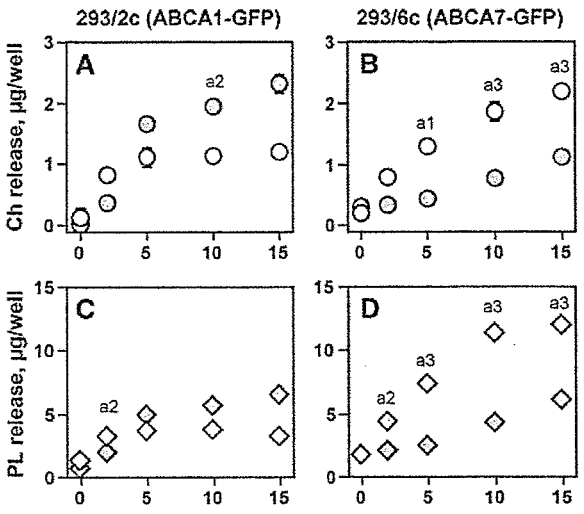

Fig. 4. Release of cholesterol (Ch; A, B) and choline-phospholipid (PL; C, D) from 293/2c (A, C) and 293/6c (B, D) cells. Cells were subcultured in six-well trays at a density of 1.0×10^6 cells/well and cultured for 48 h. After washing with buffer H, cells were incubated in 1 ml/well 0.02% BSA/DF medium containing apoA-I (open symbols) or SAA (shaded symbols) at the concentrations indicated. Cholesterol and choline-phospholipid were determined in the medium after 24 h of incubation. Results represent averages ± SD of duplicate assays in one of three independent experiments yielding similar results. Error bars are not shown when they lie within the symbols. Statistical significance for differences is indicated as a1, a2, and a3, for $P < 0.05$, 0.01, and 0.001 between apoA-I and SAA at the concentrations matched. GFP, green fluorescent protein.

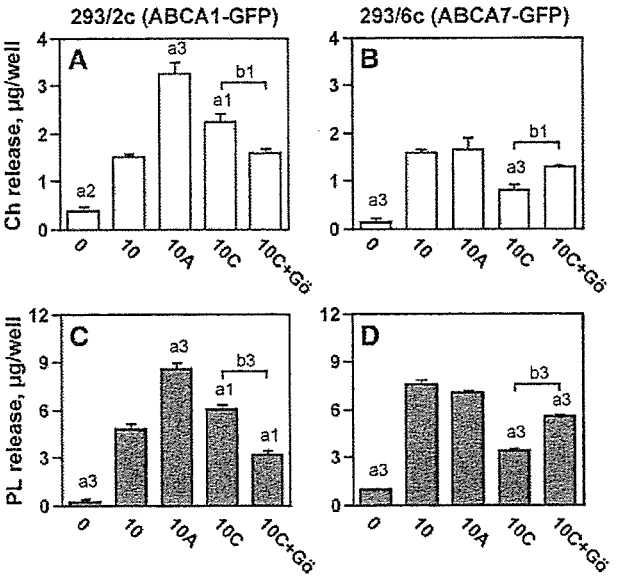

Fig. 5. Effects of dibutyryl cyclic AMP (dBcAMP), phorbol 12-myristate 13-acetate (PMA), and Gö6976 on SAA-mediated lipid release from 293/2c (A, C) and 293/6c (B, D) cells. Cells were cultured for 48 h as described for Fig. 4. The cells were then washed with buffer H and cultured in 0.02% BSA/DF medium with 10 μg/ml SAA (10) and the indicated concentration of compounds (dBcAMP, 10A; PMA, 10C; and PMA plus Gö6976, 10C+Gö) or in 0.02% BSA/DF alone (0). Lipids in the medium were determined after 24 h of incubation. Results represent averages ± SD of two or three samples in one of three independent experiments that yielded similar results. Error bars are not shown when they lie within the symbols. Statistical significance for differences is indicated as a1, a2, and a3, for $P < 0.05$, 0.01, and 0.001 against the data for 10 μg/ml SAA and as b1 and b3 for $P < 0.05$ and 0.001 between the data indicated. Ch, cholesterol; GFP, green fluorescent protein; PL, choline-phospholipid.

Negative staining electron microscopy revealed that the apoA-I-generated HDL consisted of predominantly "disc-like" particles that tend to aggregate into a rouleau formation (**Fig. 7A, C**). The SAA-generated HDL apparently contained fewer disc-like particles and had less tendency to aggregate (Fig. 7E, G). The rouleau formation was less obvious when HDL particles were prepared with sucrose (data not shown). The distribution of particle size was obtained by measuring the major axis of the particles graphically (Fig. 7B, D, F, H). The diameter peak of the apoA-I-generated HDL was larger with 293/2c (ABCA1) than with 293/6c (ABCA7). The SAA-generated HDL was larger than the apoA-I-generated HDL, consistent with the results of HPLC.

Sizes and lipid compositions of the HDL generated from 293/2c and 293/6c are listed in **Table 1**. The results obtained by two independent procedures, HPLC and ultracentrifugation, were in good agreement. Stokes diameters of the particles in HPLC were substantially larger than their major axes measured in the electron micrographs as a result of their disc-like amorphous and asymmetric shape. Almost all of the lipids were unesterified cholesterol and phospholipid, and the cholesterol-to-phospholipid ratio was less in the ABCA7-generated HDL (293/6c) than in the ABCA1-mediated HDL (293/2c). The contents of cholesteryl ester and triglyceride were both negligible in the HDLs. The level of cholesteryl ester was also negligible in the apoA-I-conditioned medium of all cells, including THP-1 and CHO-K1 (data not shown). Neither lecithin:cholesterol acyltransferase nor lipase activity was detected in the conditioned medium of 293/2c and 293/6c as well as parent HEK293 cells with or without lipid acceptors (data not shown), indicating that the differences in lipid composition or particle diameter are not caused by secondary modification of the HDL.

The electrophoretic mobility of lipoproteins generated in the SAA-conditioned medium was the same between 293/2c and 293/6c cells and slower than that generated in the apoA-I-conditioned medium (**Fig. 8**).

DISCUSSION

The results are summarized as follows. *1*) SAA interacts with the cells and induces cellular lipid release in a very similar manner to apoA-I, with respect to the phospholipid and cholesterol release profiles. *2*) Also like the apoA-I-cell interaction, the reaction requires ABCA1 or ABCA7, except that the reaction of SAA is less effective than that of apoA-I with ABCA7 in HEK293 cells. *3*) HDL generated

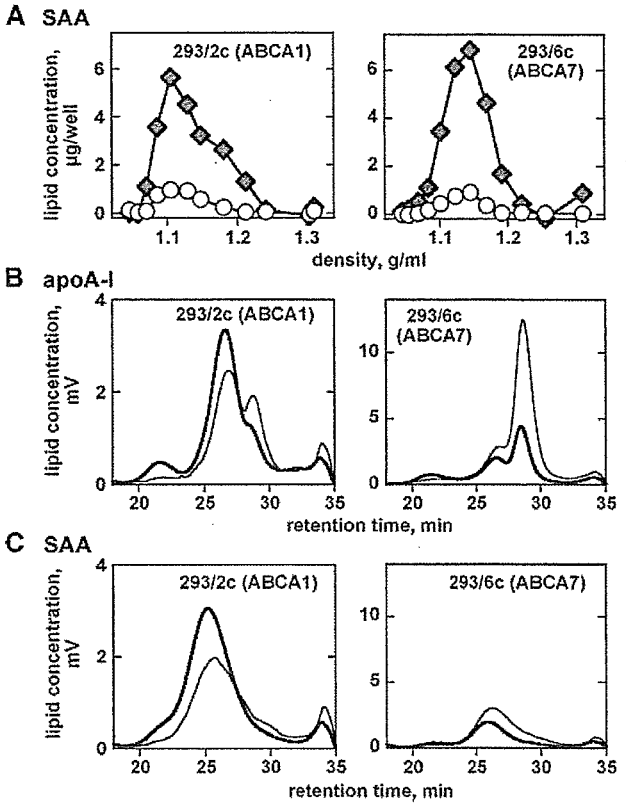

Fig. 6. Characterization of the SAA-derived HDL particles. A: Density gradient analysis of the SAA-conditioned medium of 293/2c and 293/6c cells (ABCA1 and ABCA7). Cells were cultured and stimulated with 10 µg/ml SAA. The medium was analyzed by density gradient ultracentrifugation as described in Materials and Methods. Cholesterol (open circles) and choline-phospholipid (shaded diamonds) were measured for each fraction. B, C: Size exclusion HPLC analysis of the conditioned medium of 293/2c and 293/6c cells (ABCA1 and ABCA7). Cells were cultured for 48 h as described for Fig. 4, washed with buffer H, and incubated in 0.02% BSA/DF medium with 10 µg/ml apoA-I (B) or SAA (C) for 24 h. The medium was analyzed by HPLC as described in the text, and cholesterol (thick lines) and choline-phospholipid (thin lines) were monitored online in the eluent with absorbance at 550 and 585 nm, respectively, and are expressed in mV calibrated as 30.0 nM/mV for cholesterol and 55.6 nM/mV for choline-phospholipid.

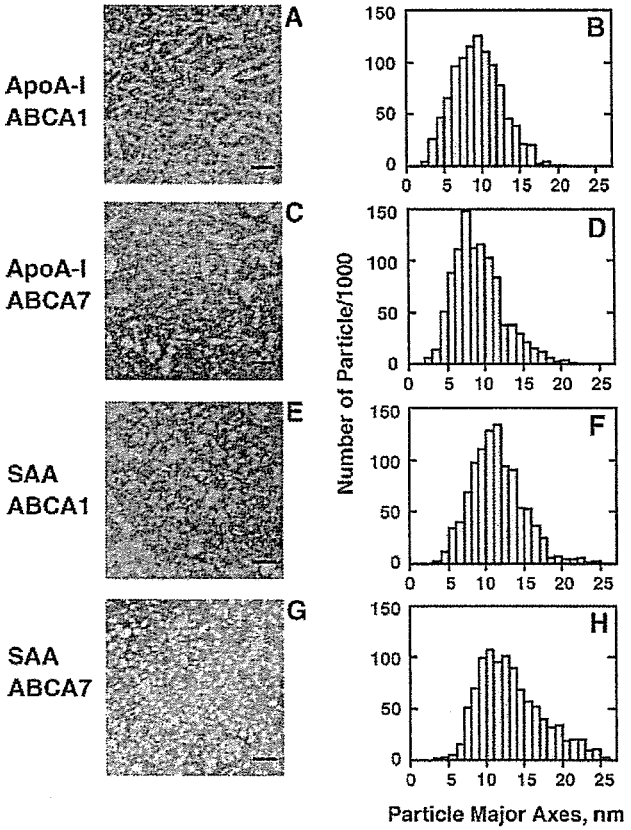

Fig. 7. Electron micrographs of negatively stained HDL particles from 293/2c and 293/6c cells. Conditioned medium was prepared as described for Fig. 6, and HDL fractions were prepared with NaBr as described in Materials and Methods. Each scale bar represents 50 nm in panels A, C, E, G. Histograms B, D, F, H represent the size distribution of HDL particles in panels A, C, E, G, respectively, based on graphic measurement of the major axes of particles using NIH Image 1.63 software.

by SAA is substantially different from that produced by apoA-I in its physicochemical properties.

HDL is generated by the ABCA1-mediated reaction with various peptides having amphiphilic α-helical segments, including helical apolipoproteins (35, 45), synthetic peptides (46, 47), and even those containing only D-amino acids (44, 48). Thus, there is no specific requirement of amino acid sequence for the biogenesis of HDL except for amphiphilic helices as a key conformation. A prediction from the secondary structure of human SAA1 indicated two amphiphilic α-helix regions (4), fulfilling this requirement. Amphiphilic segment conformation is also related to the stabilization of ABCA1 from calpain-mediated degradation (33), perhaps related to the phosphorylation of ABCA1 (43, 44). ABCA1 apparently increased upon incubation with SAA, so this protein also seems capable of inducing this effect.

SAA reproduced a profile of the apoA-I-mediated reaction. Protein kinase C activation increased the ABCA1-mediated lipid release but suppressed that mediated by ABCA7. The lipid composition of the HDL exhibited a similar difference between ABCA1 and ABCA7 by both apoA-I and SAA (27, 39). The cellular profile of apoA-I to produce cholesterol-rich HDL, cholesterol-poor HDL, and no HDL (42) is also reproduced by SAA. However, SAA induces as much lipid release as apoA-I from the ABCA1-transfected cells (293/2c) but significantly less than apoA-I from the ABCA7-transfected cells (293/6c).

It was reported recently that SAA removes cellular lipids in an ABCA1-dependent manner but also induces substantial lipid release even without ABCA1, in contrast to apoA-I (28). This is inconsistent with our present results that there was no significant lipid release observed with HEK293 cells without transfection of ABCA1 or ABCA7. The reason for this disagreement is unknown. The mass of cholesterol and choline-phospholipids in the medium

TABLE 1. Apparent size and lipid composition of the HDLs generated by apoA-I and SAA from HEK293 cells in which ABCA1 or ABCA7 was transfected and expressed (293/2c or 293/6c)

		HDL size			Ch to PL in HDL	
		Stoke's diameter, Ch/PL		Electron micrograph, peak range		
Cell	Protein	peak 1	peak 2		HPLC	Ultracentrifuge
		nm			*w/w*	
293/2c	ApoA-I	15.2/14.3	10.2/9.8	9–10	0.258	0.276
	SAA	20.2/18.2		11–12	0.254	0.305
293/6c	ApoA-I	15.3/14.4	10.4/10.0	7–8	0.100	0.137
	SAA	17.5/16.2		10–11	0.132	0.181

ApoA-I, apolipoprotein A-I; Ch, cholesterol; PL, choline-phospholipid; SAA, serum amyloid A. For the HPLC analysis, the size represents Stokes diameters of the peaks of cholesterol and choline-phospholipid based on column calibration by spherical standards (see text), and the lipid composition data were calculated by integration of the HDL peaks (see Fig. 6B, C). Peak 1 is predominant in the 293/2c-conditioned medium, and peak 2 is predominant in the 293/6c-conditioned medium when apoA-I is present (see Fig. 6B, C). For the ultracentrifugation analysis, the size represents the peak range of the histogram obtained by direct measurement of the major axes of the particles in the electron micrograph in Fig. 7, and the lipid composition data were obtained directly by measuring lipid in the HDL fraction isolated by ultracentrifugation in sucrose (see text). Lipids other than unesterified cholesterol and choline-phospholipid, such as cholesteryl ester and triglyceride, were negligible in both analyses. Data are representative of one of two independent experiments that yielded similar results.

was measured directly in our experiments, whereas in the previous report the lipid release by SAA was measured by counting radioactivity released into the medium after labeling the cell lipids and was expressed as a percentage of the count in cellular lipids. L929 cells, which express substantial levels of ABCA1 protein and release only phospholipid by apoA-I (27, 42), responded to SAA by releasing only phospholipid and no cholesterol (Fig. 2C, D), so it is likely that SAA does not induce significant ABCA1-independent lipid release. Cholesterol release from parent HEK293 and L929 cells was observed only when incubated with (2-hydroxypropyl)-β-cyclodextrin, an acceptor for diffusion-mediated cholesterol release (data not shown). Thus, we conclude that SAA induces cellular lipid release and generates HDL, dependent on ABCA1 or ABCA7.

The physicochemical properties of HDL produced by SAA were characterized in comparison with those generated by apoA-I. *1*) Sucrose density gradient ultracentrifugation data showed that the density peaks of SAA-generated HDL were higher (1.10 and 1.14 g/ml in 293/2c and 293/6c cells, respectively) than those obtained from apoA-I-generated HDL [1.08 g/ml (27)]. *2*) HPLC analysis demonstrated that HDL particles generated by SAA appeared as a single broad symmetric peak of relatively large size, whereas the apoA-I-generated HDL appeared in two distinct peaks, both of which were smaller than the SAA-generated HDL (39). *3*) Negative staining electron microscopy confirmed that the size distribution profiles of HDL were consistent with the results of HPLC and showed that rouleau formation was more obvious in the apoA-I-generated HDL than in the SAA-generated HDL. *4*) Agarose gel electrophoresis showed distinct differences in the surface charge of these particles, reflecting the isoelectric point values of apoA-I and SAA [5.40 and 6.35, respectively, calculated by the EMBL WWW Gateway to Isoelectric Point Service (http://www.embl-heidelberg.de/cgi/pi-wrapper.pl)]. These results are consistent with the finding that SAA-containing HDL particles isolated from patients with severe inflammatory disease (5) and from mice after intraperitoneal injection of LPS (3) are denser and larger than those from their normal counterparts. It should be noted that all of these physicochemical properties, including rouleau formation, are more dependent on the type of apolipoprotein than on contributing ABC transporters or the lipid composition of the generated HDL.

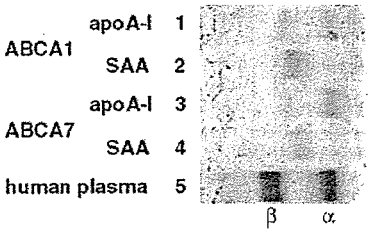

Fig. 8. Agarose gel electrophoresis of apoA-I- and SAA-containing HDL. 293/2c (ABCA1; lanes 1, 2) and 293/6c (ABCA7; lanes 3, 4) cells were incubated with 10 μg/ml apoA-I (lanes 1, 3) or SAA (lanes 2, 4) for 24 h as indicated for Fig. 6. Each conditioned medium was briefly centrifuged to remove cell debris and then processed by ultrafiltration. Medium concentrated by 20-fold was analyzed by agarose gel electrophoresis and Sudan Black staining. α and β indicate HDL and LDL, respectively, in human plasma.

SAA-containing HDL particles can also be produced by displacement of apoA-I by SAA. In vitro experiments in fact demonstrated that lipid-free SAA displaced preexisting apoA-I on HDL (5, 49). It is not known whether SAA-HDL produced by apoA-I displacement has the same properties as the SAA-generated HDL or what proportion of SAA-HDL is produced by the apoA-I displacement.

The presence of HDLs with different physicochemical properties may influence HDL metabolism. However, information from the literature is diverse. Four possible SAA receptors have been reported: FPRL1 (a G protein-coupled chemotactic receptor) (50); a receptor for advanced glycation end product (51); Tanis (a membrane selenoprotein) (52, 53); and SR-BI (10, 13). Among these,

SR-BI binds both lipid-free SAA and SAA-containing HDL and also binds apoA-I and apoA-I-containing HDL (13). SAA molecules were cointernalized with transferrin (10) in an SR-BI-dependent manner (13). SAA binding to SR-BI-expressing cells caused activation of the ERK1/2 and p38 pathway (10), which was proposed to be a mechanism for SAA-mediated cytokine production (7). These reactions were blocked by HDL, apoA-I, and amphiphilic α-helical peptides (10). These findings, however, do not clearly help us to understand the roles of SAA in HDL metabolism.

Thus, the functions of SAA and SAA-HDL are still to be determined. They may be partly responsible for host defense mechanisms. They may help to remove cholesterol from an inflammation site. Finally, they may just be a secondary protein to the inflammatory event. Studying the production mechanism of SAA-containing HDL should help us to understand the functions of SAA.

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Efflux of sphingomyelin, cholesterol, and phosphatidylcholine by ABCG1[S]

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Abstract **Cholesterol and phospholipids are essential to the body, but an excess of cholesterol or lipids is toxic and a risk factor for arteriosclerosis. ABCG1, one of the half-type ABC proteins, is thought to be involved in cholesterol homeostasis. To explore the role of ABCG1 in cholesterol homeostasis, we examined its subcellular localization and function. ABCG1 and ABCG1-K120M, a WalkerA lysine mutant, were localized to the plasma membrane in HEK293 cells stably expressing ABCG1 and formed a homodimer. A stable transformant expressing ABCG1 exhibited efflux of cholesterol and choline phospholipids in the presence of BSA, and the cholesterol efflux was enhanced by the presence of HDL, whereas cells expressing ABCG1-K120M did not, suggesting that ATP binding and/or hydrolysis is required for the efflux. Mass and TLC analyses revealed that ABCG1 and ABCA1 secrete several species of sphingomyelin (SM) and phosphatidylcholine (PC), and SMs were preferentially secreted by ABCG1, whereas PCs were preferentially secreted by ABCA1. These results suggest that ABCA1 and ABCG1 mediate the lipid efflux in different mechanisms, in which different species of phospholipids are secreted, and function coordinately in the removal of cholesterol and phospholipids from peripheral cells.**— Kobayashi, A., Y. Takanezawa, T. Hirata, Y. Shimizu, K. Misasa, N. Kioka, H. Arai, K. Ueda, and M. Matsuo. **Efflux of sphingomyelin, cholesterol, and phosphatidylcholine by ABCG1.** *J. Lipid Res.* **2006.** 47: **1791–1802.**

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Cholesterol is important to the body as a component of cellular membranes and a precursor of steroid hormones. However, excess cholesterol is a risk factor for atherosclerosis. Thus, cholesterol levels are strictly regulated by synthesis and circulation in the body. Many ABC proteins are reported to function in lipid homeostasis (1). For example, ABCG5 and ABCG8 mediate the efflux of cholesterol and sitosterol from intestine and hepatocytes into intestinal lumen and bile duct (2, 3). ABCB4 (multidrug resistance 3) is a phosphatidylcholine (PC) flippase and functions in the secretion of PC into bile duct from hepatocytes (4). ABCA1 mediates the efflux of cholesterol and phospholipids from macrophages to form HDL (5).

ABCG1 is a half-type ABC protein, with a nucleotide binding fold (NBF) in its N-terminal half and a transmembrane region in its C-terminal half. Human *ABCG1* cDNA was cloned as a gene homologous to *white* in *Drosophila* (6), a transporter of eye pigments, and many N-terminal variant forms of ABCG1 have been reported (7, 8). Other members of the ABCG subfamily form a dimer to function. For example, ABCG2 forms a homodimer to participate in multidrug resistance in breast cancer cells (9), whereas ABCG5 and ABCG8 form a heterodimer to function in the efflux of sterol from cells in the small intestine and in hepatocytes (10, 11). Cserepes et al. (12) showed that a WalkerA lysine mutant of ABCG4 inhibited the ATPase activity of ABCG1 in a dominant-negative manner and suggested that ABCG1 can heterodimerize with ABCG4, which is most homologous to ABCG1 among the ABCG subfamily. Cross-linking experiments suggested that ABCG1 forms a homodimer (13). However, it has not been clearly shown that ABCG1 functions as a homodimer.

It is thought that ABCG1 is involved in transporting cholesterol, because it is induced upon the loading of macrophages with cholesterol via a pathway of nuclear hormone receptors, liver X receptor (LXR) and retinoid X receptor (RXR) (7, 14, 15). The *ABCG1* gene is expressed in lung, brain, spleen, and macrophages. In liver, ABCG1 is expressed mainly in Kupffer cells (16). Expression of

Abbreviations: apoA-I, apolipoprotein A-I; DSP, dithiobis (succinimidylpropionate); DTBP, dimethyl 3,3′-dithiobispropionimidate-HCl; LXR, liver X receptor; NBF, nucleotide binding fold; PC, phosphatidylcholine; RXR, retinoid X receptor; SM, sphingomyelin; sulfo-NHS-biotin, sulfo-*N*-hydroxysuccinimidobiotin.

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ABCG1 was increased in macrophages from patients with Tangier disease compared with control macrophages (17). Kennedy et al. (18) reported that disruption of *ABCG1* in mice on a high-fat, high-cholesterol diet showed accumulation of both neutral lipids and phospholipids in hepatocytes and macrophages, whereas overexpression of *ABCG1* protected murine tissues from lipid accumulation. Endogenous ABCG1 is reported to localize to the perinuclear region and, in some cases, is distributed in the plasma membrane of macrophage-derived foam cells (14, 17). It was reported that ABCG1 mediates the efflux of cholesterol from cells to HDL-2 or HDL-3 but not to lipid-poor apolipoprotein A-I (apoA-I) (19) and that ABCG1 redistributes cholesterol to cell surface domains accessible for removal by HDL (13). Consistent with these reports, inhibition of ABCG1 protein expression resulted in reduced HDL-3-dependent efflux of cholesterol and phospholipids in macrophages (14). These findings suggest that ABCG1 is involved in lipid efflux in peripheral cells, like ABCA1. However, the mechanism of efflux by ABCG1 is not clear.

In this study, we investigated the subcellular localization and function of ABCG1. We demonstrated that ABCG1 localized to the plasma membrane in a HEK293 stable cell line and that ABCG1 mediates the efflux of cholesterol and phospholipids [preferentially sphingomyelin (SM)] from cells.

MATERIALS AND METHODS

Materials

Rabbit polyclonal anti-ABCG1 antibody, goat polyclonal anti-ABCG1 antibody, mouse monoclonal anti-myc antibody, goat polyclonal anti-myc antibody, and mouse monoclonal anti-FLAG antibody were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-ABCG8 antibody and mouse monoclonal anti-PentaHis antibody were obtained from Novus Biologicals and Qiagen, respectively. Rabbit polyclonal anti-FLAG and anti-BSA antibodies were purchased from Sigma. Mouse monoclonal anti-ABCA1 antibody was prepared as described previously (20). *ABCG5* and *ABCG8* cDNA were cloned from a human liver cDNA library. Dithiobis (succinimidylpropionate) (DSP), dimethyl 3,3′-dithiobispropionimidate-HCl (DTBP), and sulfo-*N*-hydroxysuccinimidobiotin (sulfo-NHS-biotin) were purchased from Pierce. Marathon cDNA libraries from fetal liver or placenta were obtained from Clontech. 8-Azido-[α-^{32}P]ATP was purchased from Affinity Labeling Technologies. Other chemicals were purchased from Sigma, Amersham Biosciences, Wako Pure Chemical Industries, and Nacalai Tesque.

Cloning of *ABCG1* cDNA

ABCG1 cDNA was cloned from a human fetal liver and placental cDNA library. The cDNA sequence cloned in this study was the same as that reported by Kennedy et al. (7) (GenBank accession number NM_207630) except for its N terminus, which corresponded to a variant reported by Chen et al. (6) (GenBank accession number X91249). A WalkerA lysine mutant *ABCG1* (ABCG1-K120M) was prepared with the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) as described by the manufacturer. The cloned cDNA was inserted into the *Not* I site of pcDNA3.1(+) (Invitrogen) to make an expression vector for pcDNA3.1(+)/ABCG1 and pcDNA3.1(+)/ABCG1-K120M, or into the *Not*I-*Xba*I site of pcDNA3.1(+)A-mycHis (Invitrogen) or pcDNA3.1(+)A-FLAG [prepared by inserting FLAG tag sequences instead of myc and His tag sequences of pcDNA3.1(+)-A-mycHis] to make an expression vector for pcDNA3.1(+)A-mycHis/ABCG1 or pcDNA3.1(+)A-FLAG/ABCG1.

Cell culture

HEK293 cells were grown in DMEM supplemented with 10% (v/v) FBS in 5% CO_2 at 37°C. THP-1 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) FBS in 5% CO_2 at 37°C. The differentiation of THP-1 monocytes into macrophages was induced with 0.2 μg/ml phorbol 12-myristrate 13-acetate (Wako Pure Chemical) for 4 days. The differentiated cells were cultured in RPMI 1640 medium and 0.2% BSA for 24 h, and ABCG1 expression was induced for 24 h by adding TO901317 (Cayman).

Establishment of a stable transformant of ABCG1

HEK293 cells were transfected with pcDNA3.1(+)/ABCG1 or pcDNA3.1(+)/ABCG1-K120M using LipofectAMINE (Invitrogen) according to the manufacturer's instructions. Cells were selected with 1 mg/ml geneticin (G418) for 2 weeks. Single colonies were isolated, and the expression of ABCG1 was examined by Western blotting and immunofluorescent staining with rabbit polyclonal anti-ABCG1 antibody.

Glycosylation of ABCG1

Digestion with endoglycosidase H and peptide *N*-glycosidase F (New England Biolabs, Beverly, MA) was done as described by the manufacturer. In brief, 20 μg of membrane protein from cells was treated with 500 units of endoglycosidase H or 0.3 units of peptide *N*-glycosidase F for 1 h at 37°C. The deglycosylated proteins were electrophoresed on a 10% SDS-polyacrylamide gel and immunodetected using rabbit polyclonal anti-ABCG1 antibody.

Immunostaining and fluorescence microscopy

Cells were cultured on glass cover slips, fixed with 4% paraformaldehyde in PBS$^+$ (phosphate-buffered saline containing 0.1 g/l $CaCl_2$ and $MgCl_26H_2O$), and permeabilized with 0.4% Triton X-100 in PBS$^+$ for 5 min. To diminish the nonspecific binding of antibodies, the cells were incubated in 10% goat serum in PBS$^+$. Cells were incubated for 1 h with rabbit polyclonal anti-ABCG1 antibody diluted 1:500 in PBS$^+$ containing 10% goat serum and then incubated with fluorescent Alexa 488-conjugated anti-rabbit IgG (Molecular Probes) for 1 h. Cells were directly viewed with a 63× Plan-Neofluar oil-immersion objective using a Zeiss confocal microscope (LSM5 Pascal).

Biotinylation of cell surface proteins

Cells were washed with ice-cold PBS$^+$ and incubated with 0.5 mg/ml sulfo-NHS-biotin solubilized in PBS$^+$ for 30 min on ice in the dark. Cells were washed with PBS$^+$ to remove unbound sulfo-NHS-biotin and lysed in RIPA buffer [20 mM Tris-Cl (pH 7.5), 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate] containing protease inhibitors [100 μg/ml (*p*-amidinophenyl)-methanesulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). ImmunoPure Immobilized Monomeric Avidin Gel (Pierce) was added to the cell lysate to precipitate the biotinylated proteins. The biotinylated proteins were electrophoresed on a 10% SDS-polyacrylamide gel and immunodetected.

Coimmunoprecipitation

After 48 h of transfection with pcDNA3.1(+)A-mycHis/ABCG1 or pcDNA3.1(+)A-FLAG/ABCG1, cells were washed with PBS and lysed in Nonidet P-40 lysis buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, and 1% Nonidet P-40] containing protease inhibitors. The lysates were incubated with antibodies and immunoprecipitated with protein G-Sepharose 4B Fast Flow (Sigma). The immunoprecipitated proteins were washed with Nonidet P-40 lysis buffer and electrophoresed on a 10% SDS-polyacrylamide gel.

Cross-linking

Cells were washed with cold PBS and incubated with 250 μM DSP or DTBP at room temperature for 30 min. The cross-linking reaction was terminated by the addition of Tris-Cl buffer (pH 7.5) to 20 mM, and cells were incubated at room temperature for 15 min. Cells were washed with PBS and lysed in Nonidet P-40 lysis buffer. Samples were denatured in SDS sample buffer with or without DTT, electrophoresed on a 7% SDS-polyacrylamide gel, and immunodetected.

Photoaffinity labeling

Membranes (20 μg of proteins) from HEK293 cells, prepared as described previously (21), were incubated with 50 μM 8-azido-[α-^{32}P]ATP in 3 μl of TEM buffer [40 mM Tris-Cl (pH 7.5), 0.1 mM EGTA, and 1 mM $MgCl_2$] containing 2 mM ouabain for 10 min on ice. Proteins were ultraviolet light-irradiated for 3 min (at 254 nm, 5.5 mW/cm^2) on ice. To remove free 8-azido-[α-^{32}P]ATP, ice-cold TEM buffer was added to the sample and the supernatant was removed after centrifugation (15,000 *g*, 5 min, 2°C). Pellets were resuspended in 100 μl of RIPA buffer containing 100 μg/ml (*p*-amidinophenyl)methanesulfonyl fluoride, and membrane proteins were solubilized for 30 min at 4°C. After centrifugation for 15 min at 15,000 *g*, the lysates were incubated with antibodies and immunoprecipitated with protein G-Sepharose 4B Fast Flow (Sigma). The immunoprecipitated proteins were washed with RIPA buffer, and samples were electrophoresed on a 10% SDS-polyacrylamide gel and autoradiographed.

Fractional lipid release assay

Cells were subcultured on 12-well plates at a density of 5.0 × 10^5 cells. After incubation for 24 h, the cells were labeled with 2 μCi/ml [^{3}H]cholesterol or [^{3}H]choline for 24 h in DMEM containing 10% FBS. The cells were washed with fresh medium and incubated with DMEM containing 0.02% BSA in the absence or presence of 10 μg/ml apoA-I (Calbiochem) or 20 μg/ml HDL (Calbiochem) for 4 h. For cholesterol efflux, the medium was collected and the cells were lysed with 0.1 N NaOH and 0.1% SDS. For phospholipid efflux, phospholipids were extracted from the medium with chloroform-methanol (2:1) or from the cells with hexane-isopropanol (3:2). PC and SM were separated by TLC on silica gel 60 plates (Merck) developed in chloroform-methanol-acetic acid-water (60:30:10:5). The radioactivity was counted by liquid scintillation counting.

Cellular lipid release assay

Cells were subcultured on six-well plates at a density of 1.0 × 10^6 cells. After incubation for 24 h, the cells were washed with fresh medium and incubated with DMEM containing 0.02% BSA in the absence or presence of 10 μg/ml apoA-I. The lipid content in the medium was determined after 24 or 48 h of incubation as described previously (22).

Mass spectrometric analysis

Cells were subcultured on six-well plates at a density of 1.0 × 10^6 cells. After incubation for 24 h, the cells were washed with fresh medium and incubated with DMEM containing 0.02% BSA in the absence or presence of 10 μg/ml apoA-I for 48 h. The medium was centrifuged for 15 min at 7,000 *g* to remove cell debris twice. The lipids were extracted from 12 ml of medium by the method of Bligh and Dyer (23) after the addition of di-14:1 PC as an internal standard. The content of choline phospholipids in the medium was analyzed by MS. Mass spectrometric analyses were performed with a triple quadrupole instrument model Quattro micro (Micromass, Manchester, UK) equipped with an electrospray source as described previously (24). The samples were provided by the UltiMate high-performance liquid chromatography system (LC Packings, San Francisco, CA) into the electrospray interface at a flow rate of 4 μl/min in a solvent system of acetonitrile-methanol-water (2:3:1) containing 0.1% ammonium formate (pH 6.4). The mass spectrometer was operated in the positive and negative scan modes. The flow rate of the nitrogen drying gas was 12 l/min at 80°C. The capillary and cone voltages were set at 3.7 kV and 30 V, respectively, argon at 3–4 × 10^{-4} Torr was used as the collision gas, and a collision energy of 30–40 V was used to obtain fragment ions for precursor ions. The relationship between peak height and amount of SM was examined using bovine brain SM (Avanti).

Statistical analysis

Values are presented as means ± SD. Statistical significance was determined by Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Expression of human ABCG1 protein in HEK293 cells

To analyze the subcellular localization and function of human ABCG1, the ABCG1 expression vector was introduced into HEK293 cells, and a cell line stably expressing ABCG1 (HEK/ABCG1) was established. HEK293 cells stably expressing ABCG1-K120M (HEK/ABCG1-K120M), a WalkerA lysine mutant, were also established. Both cell lines expressed similar amounts of ABCG1 migrating as ~60 kDa proteins on SDS-PAGE (**Fig. 1A**, lanes 4, 5). THP-1 cells, differentiated by phorbol 12-myristrate, faintly expressed ABCG1 migrating at ~60 kDa, and ABCG1 was induced by TO901317, a LXR ligand (Fig. 1A, lanes 1, 2). The amount of ABCG1 expressed in HEK/ABCG1 cells was comparable to that in THP-1 cells induced by TO901317. Other members of the ABCG subfamily (ABCG2, ABCG5, and ABCG8) have been reported to be glycosylated (10, 25). Indeed, ABCG5, expressed without ABCG8 and modified with high-mannose-type *N*-linked oligosaccharide, migrated faster after treatment with *N*-glycosidase F and endoglycosidase H (Fig. 1B, lanes 8–10), as reported (10). However, the migration of ABCG1, expressed in both THP-1 and HEK/ABCG1 cells, was not changed by treatment with either glycosidase (Fig. 1B, lanes 1–3, 5–7).

Localization of ABCG1 in plasma membrane

ABCG1 was reported to be distributed mainly in the perinuclear region and only partly in the plasma mem-

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