Unlike ABCA1, there is no consensus sequence for glycosylation in other extracytosolic loops. Lysosome-related organelles including the lamellar body have proteolytic enzymes in their lumen, and therefore hABCA3 may be cleaved at its lumenal loop after the second N-linked glycosylation site (asparadine-140) in vesicular structures as was reported for the yeast vacuolar ABC transporter Ycf1p [22,23].

Recently, we showed that human ABCA7 expressed in HEK293 cells is localized at the plasma membrane, and the first extracellular domain is exposed to the extracellular space [24,25] as is the case for ABCA1 [25,26]. ABCA7 showed apoA-I-dependent cholesterol and phospholipid release [11,24] as ABCA1 [9,26]. On the other hand, ABCA4 associates with intracellular vesicles when expressed in COS-1 cells [27]. ABCA4 is expressed in photoreceptors and transports retinal-phosphatidylethanolamine complexes across the photoreceptor disk membrane of rods [10,28,29]. ABCA3 is not targeted to the plasma membrane either, but to the membrane of intracellular vesicular structures when stably expressed in HEK293 cells. This is consistent with previous observations in which ABCA3 was mainly localized at intracellular vesicles when transiently expressed in the human adenocarcinoma cell lines A549 or NCI H441 [7]. The size (0.6–1μm in diameter) of the vesicular structures is also comparable with that of lamellar bodies in alveolar type II cells. In conclusion, ABCA3 may be involved in dynamics of cholesterol and possibly also phospholipid, and the biogenesis of lamellar body-like structures.

Acknowledgments

We thank Drs. Toyoshi Fujimoto (Nagoya Univ.) and Masayuki Murata (Tokyo Univ.), and Mr. Fumiyoshi Ishidate (Carl Zeiss Japan) for helpful discussion and technical assistance. This work was supported by Grant-in-Aid for Scientific research and Creative Scientific Research 15GS0301 from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Bio-oriented Technology Research Advancement Institution (BRAIN).

References

[1] G. Schmitz, G. Muller, Structure and function of lamellar bodies, lipid–protein complexes involved in storage and secretion of cellular lipids, J. Lipid Res. 32 (1991) 1539–1570.
[2] D.F. Cutler, Introduction: lysosome-related organelles, Semin. Cell Dev. Biol. 13 (2002) 261–262.
[3] T.E. Weaver, C.-L. Na, M. Stahlman, Biogenesis of lamellar bodies, lysosome-related organelles involved in storage and secretion of pulmonary surfactant, Semin. Cell Dev. Biol. 13 (2002) 263–270.
[4] T.D. Connors, T.J. Van Raay, L.R. Petry, K.W. Klinger, G.M. Landes, T.C. Burn, The cloning of a human ABC gene (ABC3) mapping to chromosome 16p13.3, Genomics 39 (1997) 231–234.
[5] N. Klugbauer, F. Hofmann, Primary structure of a novel ABC transporter with a chromosomal localization on the band encoding the multidrug resistance-associated protein, FEBS Lett. 391 (1996) 61–65.
[6] G. Yamano, H. Funahashi, O. Kawanami, L.X. Zhao, N. Ban, Y. Uchida, T. Morohoshi, J. Ogawa, S. Shioda, N. Inagaki, ABCA3 is a lamellar body membrane protein in human lung alveolar type II cells, FEBS Lett. 508 (2001) 221–225.
[7] S. Mulugeta, J.M. Gray, K.L. Notarfrancesco, L.W. Gonzales, M. Koval, S.I. Feinstein, P.L. Ballard, A.B. Fisher, H. Shuman, Identification of LBM180, a lamellar body limiting membrane protein of alveolar type II cells, as the ABC transporter protein ABCA3, J. Biol. Chem. 277 (2002) 22147–22155.
[8] S. Shulenin, L.M. Nogee, T. Annilo, S.E. Wert, J.A. Whitsett, M. Dean, ABCA3 gene mutations in newborns with fatal surfactant deficiency, N. Engl. J. Med. 350 (2004) 1296–1303.
[9] A.R. Tanaka, S. Abe-Dohmae, T. Ohnishi, R. Aoki, G. Morinaga, K.I. Okuhira, Y. Ikeda, F. Kano, M. Matsuo, N. Kioka, T. Amachi, M. Murata, S. Yokoyama, K. Ueda, Effects of mutations of ABCA1 in the first extracellular domain on subcellular trafficking and ATP binding/hydrolysis, J. Biol. Chem. 278 (2003) 8815–8819.
[10] H. Sun, R. Molday, J. Nathans, Retinal stimulates ATP hydrolysis by purified and reconstituted ABCR, the photoreceptor-specific ATP-binding cassette transporter responsible for Stargardt disease, J. Biol. Chem. 274 (1999) 8269–8281.
[11] S. Abe-Dohmae, Y. Ikeda, M. Matsuo, M. Hayashi, K. Okuhira, K. Ueda, S. Yokoyama, Human ABCA7 supports apolipoprotein-mediated release of cellular cholesterol and phospholipid to generate high density lipoprotein, J. Biol. Chem. 279 (2004) 604–611.
[12] Y. Takada, K. Yamada, Y. Taguchi, K. Kino, M. Matsuo, S.J. Tucker, T. Komano, T. Amachi, K. Ueda, Non-equivalent cooperation between the two nucleotide-binding folds of P-glycoprotein, Biochim. Biophys. Acta 1373 (1998) 131–136.
[13] K. Ueda, N. Inagaki, S. Seino, MgADP antagonism to Mg-independent ATP binding of the sulfonylurea receptor SUR1, J. Biol. Chem. 272 (1997) 22983–22986.
[14] Y. Taguchi, A. Yoshida, Y. Takada, T. Komano, K. Ueda, Anticancer drugs and glutathione stimulate vanadate-induced trapping of nucleotide in multidrug resistance-associated protein (MRP), FEBS Lett. 401 (1997) 11–14.
[15] I.L. Urbatsch, B. Sankaran, J. Weber, A.E. Senior, P-glycoprotein is stably inhibited by vanadate-induced trapping of nucleotide at a single catalytic site, J. Biol. Chem. 270 (1995) 19383–19390.
[16] K. Hashimoto, T. Uchiumi, T. Konno, T. Ebihara, T. Nakamura, M. Wada, S. Sakisaka, F. Maniwa, T. Amachi, K. Ueda, M. Kuwano, Trafficking and functional defects by mutations of the ATP-binding domains in MRP2 in patients with Dubin–Johnson syndrome, Hepatology 36 (2002) 1236–1245.
[17] J. Cai, R. Daoud, O. Alqawi, E. Georges, J. Pelletier, P. Gros, Nucleotide binding and nucleotide hydrolysis properties of the ABC transporter MRP6 (ABCC6), Biochemistry 41 (2002) 8058–8067.
[18] U. Klein, G. Gimpl, F. Fahrenholz, Alteration of the myometrial plasma membrane cholesterol content with beta-cyclodextrin modulates the binding affinity of the oxytocin receptor, Biochemistry 34 (1995) 13784–13793.
[19] E.P. Kilsdonk, P.G. Yancey, G.W. Stoudt, F.W. Bangerter, W.J. Johnson, M.C. Phillips, G.H. Rothblat, Cellular cholesterol efflux mediated by cyclodextrins, J. Biol. Chem. 270 (1995) 17250–17256.
[20] P.G. Yancey, W.V. Rodrigueza, E.P.C. Kilsdonk, G.W. Stoudt, W.J. Johnson, M.C. Phillips, G.H. Rothblat, Cellular cholesterol

efflux mediated by cyclodextrins. Demonstration of kinetic pools and mechanism efflux, J. Biol. Chem. 271 (1996) 16026–16034.
[21] A. Garrigues, A.E. Escargueil, S. Orlowski, The multidrug transporter, P-glycoprotein, actively mediates cholesterol redistribution in the cell membrane, Proc. Natl. Acad. Sci. USA 99 (2002) 10347–10352.
[22] D.L. Mason, S. Michaelis, Requirement of the N-terminal extension for vacuolar trafficking and transport activity of yeast Ycf1p, an ATP-binding cassette transporter, Mol. Biol. Cell 13 (2002) 4443–4455.
[23] D.L. Mason, M.P. Mallampalli, G. Huyer, S. Michaelis, A region within a lumenal loop of *Saccharomyces cerevisiae* Ycf1p directs proteolytic processing and substrate specificity, Eukaryot. Cell 2 (2003) 588–598.
[24] Y. Ikeda, S. Abe-Dohmae, Y. Munehira, R. Aoki, S. Kawamoto, A. Furuya, K. Shitara, T. Amachi, N. Kioka, M. Matsuo, Posttranscriptional regulation of human ABCA7 and its function for the apoA-I-dependent lipid release, Biochem. Biophys. Res. Commun. 311 (2003) 313–318.
[25] M.L. Fitzgerald, A.J. Mendez, K.J. Moore, L.P. Andersson, H.A. Panjeton, M.W. Freeman, ABCA1 contains an N-terminal signal-anchor sequence that translocates the protein's first hydrophilic domain to the exoplasmic space, J. Biol. Chem. 276 (2001) 15137–15145.
[26] A.R. Tanaka, Y. Ikeda, S. Abe-Dohmae, R. Arakawa, K. Sadanami, A. Kidera, S. Nakagawa, T. Nagase, R. Aoki, N. Kioka, T. Amachi, S. Yokoyama, K. Ueda, Human ABCA1 contains a large amino-terminal extracellular domain homologous to an epitope of Sjogren's syndrome, Biochem. Biophys. Res. Commun. 283 (2001) 1019–1025.
[27] J. Ahn, S. Beharry, L.L. Molday, R.S. Molday, Functional interaction between the two halves of the photoreceptor-specific ATP binding cassette protein ABCR (ABCA4). Evidence for a non-exchangeable ADP in the first nucleotide binding domain, J. Biol. Chem. 278 (2003) 39600–39608.
[28] J. Weng, N.L. Mata, S.M. Azarian, R.T. Tzekov, D.G. Birch, G.H. Travis, Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in abcr knockout mice, Cell 98 (1999) 13–23.
[29] R. Allikmets, Simple and complex ABCR: genetic predisposition to retinal disease, Am. J. Hum. Genet. 67 (2000) 793–799.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 279, No. 1, Issue of January 2, pp. 604–611, 2004
Printed in U.S.A.

Human ABCA7 Supports Apolipoprotein-mediated Release of Cellular Cholesterol and Phospholipid to Generate High Density Lipoprotein*

Received for publication, September 5, 2003, and in revised form, October 13, 2003
Published, JBC Papers in Press, October 21, 2003, DOI 10.1074/jbc.M309888200

Sumiko Abe-Dohmae‡, Yuika Ikeda§, Michinori Matsuo§, Michi Hayashi‡, Kei-ichiro Okuhira‡, Kazumitsu Ueda§, and Shinji Yokoyama‡¶

From ‡Biochemistry, Molecular Biology and Metabolism 1, Nagoya City University Graduate School of Medical Sciences, Nagoya 467-8601 and the §Laboratory of Cellular Biochemistry, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

Apolipoprotein-mediated release of cellular cholesterol and phospholipids was induced in HEK293 cells by expressing human ATP-binding cassette transporter A7 (ABCA7) and ABC transporter A1 (ABCA1) proteins, whether transient or stable, to generate cholesterol-rich high density lipoprotein (HDL). Green fluorescent protein (GFP) attached at their C termini did not influence the lipid release reactions. Transfected ABCA7-GFP induced apolipoprotein-mediated assembly of cholesterol-containing HDL also in L929 cells, which otherwise generate only cholesterol-deficient HDL with their endogenous ABCA1. Time-dependent release of cholesterol and phospholipid by apolipoprotein A (apoA)-I was parallel both with ABCA1 and with ABCA7 when highly expressed in HEK293 cells, but dose-dependent profiles of lipid release on apoA-I and apoA-II were somewhat different between ABCA1 and ABCA7. Analyses of the stable clones with ABCA1-GFP (293/2c) and ABCA7-GFP (293/6c) by using the same vector indicated some differences in regulation of their activities by protein kinase modulators. Dibutyryl cyclic AMP increased ABCA1-GFP and the release of cholesterol and phospholipid in 293/2c but increased neither ABCA7-GFP nor the lipid release in 293/6c. Expression of ABCA1-GFP- and apoA-I-mediated lipid release were enhanced in parallel by phorbol 12-myristate 13-acetate (PMA) in 293/2c cells. In contrast, the same treatment of 293/6c increased ABCA7-GFP, but apoA-I-mediated lipid release was significantly suppressed. Despite these different responses to PMA, all of the effects of PMA were reversed by a specific protein kinase C inhibitor Gö6976, suggesting that the changes were in fact due to protein kinase C activation. A thiol protease inhibitor, *N*-acetyl-Leu-Leu-norleucinal, increased the protein levels of ABCA1-GFP in 293/2c and ABCA7-GFP in 293/6c, indicating their common degradation pathway. The data indicated that human ABCA7 would compensate the function of ABCA1 for release of cell cholesterol in a certain condition(s), but post-transcriptional regulation of their activity is different.

Cholesterol is essential for all animal cells as a membrane constituent for regulation of its general physicochemical properties and functions of specific domains. Cellular cholesterol can be derived by *de novo* synthesis or externally supplied via the uptake of cholesterol-containing lipoprotein particles. In contrast, most of the cells are unable to catabolize cholesterol so that cholesterol molecules must be removed from the cells and transported to the liver for their conversion to bile acids as a major exit route of the body cholesterol. Thus cholesterol transport from the peripheral cells to the liver is an essential part of cholesterol homeostasis, both for cells and for the body. High density lipoprotein (HDL)[1] is believed to play a central role in this pathway.

Lipid-free apolipoproteins with amphiphilic α-helical segments were demonstrated to remove cellular lipids to generate cholesterol-containing HDL (1, 2). This reaction was shown deficient in fibroblasts from patients with familial HDL deficiency, Tangier disease (3, 4), and therefore found essential for generation of plasma HDL. Mutations were identified in one of the members of ATP-binding cassette (ABC) transporter superfamily, ABC transporter A1 (ABCA1), as the cause of Tangier disease and other genetic HDL deficiencies (5–9) so that a role of this protein in the generation of HDL by apolipoprotein-cell interaction became the focus of the HDL research. ABC transporter G1 (ABCG1), an ABC transporter protein of a "half-size" structure family, has also been reported to regulate the apolipoprotein A (apoA)-I-mediated lipid release from lipid-laden macrophages (10). However, it is unclear whether this protein can generate HDL in the absence of ABCA1.

ABC transporter A7 (ABCA7) is another member of the same ABCA subfamily of "full-size" transporter as ABCA1, and its cDNA has been cloned from the human macrophage and spleen cDNA libraries exhibiting high homology to other human ABC transporters (11). ABCA7 has also been identified as the autoantigen SS-N, an epitope of Sjögren's syndrome, which was found homologous to the putative first extracellular domain of ABCA1 (12). ABCA7 mRNA and protein were induced in differentiated macrophages from human peripheral monocytes, and it was apparently expressed inversely to the cellular cholesterol level (11). Although an exact role of ABCA7 in cellular cholesterol homeostasis is unknown, it may play a relevant role

* This work was supported in part by grants-in-aids from the Ministry of Education, Culture, Science and Technology and Ministry of Welfare, Health and Labor of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 81-52-853-8139; Fax: 81-52-841-3480; E-mail: syokoyam@med.nagoya-cu.ac.jp.

[1] The abbreviations used are: HDL, high density lipoprotein; ABC, ATP-binding cassette; ABCA1, ABC transporter A1; ABCG1, ABC transporter G1; ABCA7, ABC transporter A7; GFP, green fluorescent protein; DF, Dulbecco's modified Eagle's medium and Ham's F12 medium; FCS, fetal calf serum; apoA, apolipoprotein A; BSA, bovine serum albumin; lysoPC, lysophosphatidylcholine; dBcAMP, dibutyryl cyclic AMP; PMA, phorbol 12-myristate 13-acetate; ALLN, *N*-acetyl-Leu-Leu-norleucinal; PKC, protein kinase C; PKA, cAMP-dependent protein kinase.

TABLE I

ApoA-I-mediated lipid release from parent HEK293 cells treated with protein kinase stimulants

Cells were subcultured in 6-well trays at a density of 1.0×10^6 cell/well and incubated for 48 h. The cells were washed with buffer H, and 1 ml/well of 0.1 % BSA-DF containing apoA-I (10 μg/mL), dBcAMP (300 μM) and PMA (320 nM) was added as indicated. Medium was collected after 24 h for cholesterol (Ch) and phospholipid (PL) analysis (μg/well). Results shown are the average and variation for two samples.

apoA-I		+	+	+	+
dBcAMP			+		+
PMA				+	+
Ch	0.02 ± 0.01	−0.01 ± 0.03	0.05 ± 0.03	0.02 ± 0.00	0.02 ± 0.01
PL	0.53 ± 0.00	0.62 ± 0.12	0.69 ± 0.07	0.62 ± 0.07	0.59 ± 0.03

in regulation of cholesterol turnover in some specific cells such as macrophages.

Recently, it was reported that mouse ABCA7 promotes apoA-I-mediated phospholipid release but failed to release cholesterol (13). Here we report that human ABCA7 mediates apolipoprotein-dependent generation of HDL by releasing both cellular cholesterol and phospholipid even in the absence of ABCA1. Although the reaction is largely similar to that of human ABCA1, apparent affinity of apolipoproteins for the cells is different. Regulations of its expression and activity by protein kinase activators are also different from those of ABCA1.

EXPERIMENTAL PROCEDURES

DNA Construction and Transfection—Full-length cDNAs for human ABCA1 and ABCA7 were cloned as described previously (12). They were introduced to pcDNA3.1/Hygro (Invitrogen), pCMV6c, and pEGFP-N1 (Clontech) to obtain constructs for the proteins without or with green fluorescent protein (GFP) at their C terminus. All the vectors have an immediate early promoter of cytomegalovirus promoter for expression of cDNA. HEK293 and L929 cells were obtained from Health Science Research Resources Bank and maintained in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF) supplemented with 10% (v/v) of fetal calf serum (FCS, Invitrogen) under a humidified atmosphere of 5% CO_2, 95% air at 37 °C. cDNAs were transfected with LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's instruction. Cells to which the cDNAs were permanently introduced were selected with G418, and clones with high level expression of the fusion proteins were further selected according to the fluorescent intensity with GFP.

Detection of Expressed Proteins—Western blotting was carried out with an anti-ABCA1 antiserum (14, 15), a rat polyclonal anti-ABCA7 antibody, and an anti-GFP antibody (Santa Cruz Biotechnology) to indicate the expression levels of the proteins. To generate the anti-ABCA7 antibody, the putative first extracellular domain of human ABCA7, amino acids 45-459, was expressed as a fusion protein with His tag at the C terminus in *Escherichia coli*, purified by Ni^{++} chromatography (Qiagen),[2] and used for immunization. Expression levels of ABCA1-GFP protein and ABCA7-GFP protein were also measured *in situ* with an FL600 fluorescent plate reader (Bio-Tek Inc.). Cellular fluorescence was measured as fluorescent intensity per unit area. GFP-derived derived fluorescence was calculated by subtracting background. Intracellular localization of GFP-containing protein was examined by their fluorescence images obtained by using an Axiovert microscope (Carl Zeiss) equipped with a MicroRadiance confocal laser scanning microscope (Bio-Rad).

Apolipoproteins—ApoA-I and apoA-II were isolated from human plasma HDL fraction (density 1.09 - 1.21) and stored at −80 °C until use as described previously (16, 17). Stock solutions (1 mg/ml) were prepared and stored at 4 °C as described previously (18).

Cellular Lipid Release Assay—Cells were subcultured in 6-well trays (TPP catalogue number 92406) at a density of 1.0×10^6 cells/well with 10% FCS-DF medium. After a 48-h incubation, the cells were washed once with buffer H (Hank's balanced salt solution containing 20 mM HEPES-KOH (pH 7.5) and 14 mM glucose), and incubated in 1 ml/well of DF medium containing 0.02% bovine serum albumin (BSA) and lipid acceptors (apoA-I, apoA-II, and 2-hydroxypropyl-β-cyclodextrin). Buffer H supplemented with amino acids (Invitrogen catalogue number 11140-50) was used instead of DF medium in some experiments to reduce the endogenous fluorescence background in DF medium. Lipid content in the medium and cells was determined after the indicated incubation times. Procedures for lipid extraction and enzymatic assays for cholesterol and choline-phospholipids were described previously (18). Enzyme assay in combination with lysophospholipase, glycerophosphorylcholine phosphodiesterase, and choline oxidase (19) was also applied to evaluate the level of lysophosphatidylcholine (lysoPC), a causal molecule for background in choline-phospholipid assay.

Density Gradient Analysis—Cells were subcultured in 100-mm dishes (TPP catalogue number 93100) at a density of 6.0×10^6 cells/dish, cultured as above and stimulated with 5 ml/dish of DF medium containing 0.1% BSA and 10 μg/ml apoA-I for 24 h. Medium from two dishes was combined and centrifuged to remove cell debris, and 8 ml of the supernatant was processed for sucrose density gradient ultracentrifuge (1). The solution was collected from the bottom into 13 fractions. The contents of cholesterol and choline-phospholipids as well as the density were determined for each fraction.

Statistical Analysis—Data were analyzed by one-way analysis of variance followed by Scheffé's test. A p value less than 0.05 was accepted as statistically significant.

RESULTS

Parent HEK293 cells did not respond to apolipoproteins to release either cholesterol or phospholipid (Table I). Treatment with dibutyryl cyclic AMP (dBcAMP) and phorbol 12-myristate 13-acetate (PMA) with or prior to apoA-I stimulation had no effect either (Table I and data not shown). Transient expression of ABCA1 cDNA and ABCA7 cDNA in HEK293 cells resulted in apoA-I-mediated release of both cholesterol and phospholipid in a dose-dependent manner (Fig. 1), and attachment of GFP to the C terminus of ABCA7 or ABCA1 did not influence these lipid releases (see below). For further investigation of the functions of ABCA7, we therefore obtained stable clones expressing high levels of ABCA1-GFP protein and ABCA7-GFP protein.

Release of cholesterol and phospholipid by incubation with apoA-I was demonstrated at least in three independent clones highly expressing ABCA1-GFP and also three clones expressing ABCA7-GFP, indicating that the reaction is not clone-specific but cDNA-specific. Western blotting data of parent HEK293 cells, the cells with or without transient expression of ABCA1 and ABCA7, and ABCA1-GFP-expressing clone (293/2c) and ABCA7-GFP-expressing clone (293/6c) are shown in Fig. 2. Neither ABCA1 nor ABCA7 protein was detected in parent HEK293 cells. ABCA1- and ABCA7-containing bands were detected at the position consistent with those of GFP-attached molecules in 293/2c and 293/6c cells, as ABCA1-GFP and ABCA7-GFP at 260–270 and 240–260 kDa, respectively (Fig. 2, *C–E*). ABCA1 protein expression level per cell protein in 293/2c cells was estimated as about 30-fold of that in human fibroblast WI38 and mouse fibroblast L929 by Western blotting analysis (data not shown). GFP protein expression was ~1:3 between 293/2c and 293/6c cells based on Western blotting analysis results (Fig. 2*C*) and measurement of fluorescence (see Figs. 7*C* and 9*C*, compare the group 0). Confocal microscopic analysis revealed that ABCA1-GFP (20) and ABCA7-GFP (Fig. 2*F*) were localized mainly in plasma membrane, as was recently reported in CHO cells stably expressing rat ABCA7 (21) and in HEK293 cells transiently expressing mouse ABCA7 (13).

Typical profiles of lipid release by apoA-I and apoA-II from 293/2c and 293/6c cells are shown in Fig. 3. LysoPC assay

[2] R. Aoki and K. Ueda, unpublished results.

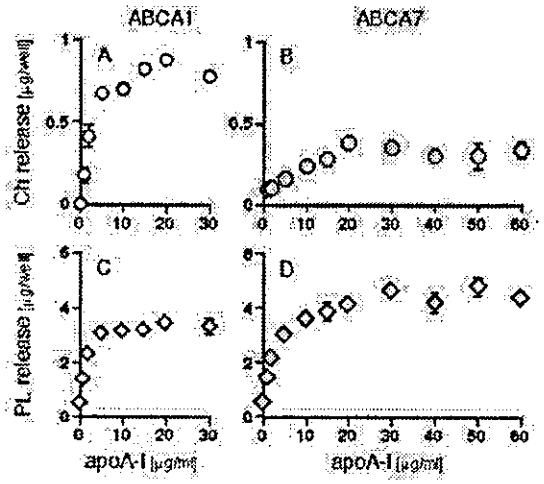

FIG. 1. **ApoA-I-mediated release of cholesterol (*Ch*, *A* and *B*) and choline-phospholipids (*PL*, *C* and *D*) from HEK293 cells transiently expressing ABCA1 cDNA (*A* and *C*) and ABCA7 cDNA (*B* and *D*).** Parent HEK293 cells were subcultured in 6-well trays at a density of 1.0×10^6 cell/well and incubated for 24 h. The cells were transfected with 1.0 μg/well of ABCA1/pcDNA3.1/Hygro (*A* and *C*) and ABCA7/pCMV6c (*B* and *D*). After a 24-h incubation, the cells were washed with buffer II (see "Experimental Procedures"), 1 ml/well of 0.02% BSA-DF containing various concentrations of apoA-I as indicated. Medium was collected after 24 h for lipid analysis. Results shown are the average and variation for two samples. *Error bars* are not shown when found to lie within the symbols. The amounts of total cellular cholesterol and choline-phospholipids in the cells at the starting time of apoA-I-incubation (48 h after subculture) were 12.0 ± 0.5 and 92.3 ± 3.3 μg/well in ABCA1/pcDNA3.1/Hygro-transfected cells, and 12.8 ± 0.4 and 105.5 ± 1.4 μg/well in ABCA7/pCMV6c-transfected cells, respectively (average ± S.D. for six samples).

confirmed that most of the choline-phospholipids released to the medium in the absence of apolipoproteins was lysoPC and that its level was independent of apolipoprotein concentration (data not shown). The dose-dependent curve of the reaction by apoA-II was similar to that of apoA-I with respect to molar concentration of the proteins for both cholesterol and choline-phospholipid (Fig. 3). These results indicated that ABCA7 directly promotes both cholesterol and phospholipid efflux to apoA-I, just as ABCA1 does. The releases of phospholipid and cholesterol appear parallel by increasing concentrations of apoA-I and apoA-II in 293/2c (ABCA1-GFP) and 293/6c (ABCA7-GFP). The lipid release seems to reach the maximum at lower concentration of apolipoprotein with the ABCA1-expresing cells (293/2c) than the ABCA7-expressing cells (293/6c). The EC_{50} for the apoa-I mediated release of cholesterol and phospholipid from 293/6c cells was ~4.5 and 4.2 μg/ml (0.16 and 0.15 μM), respectively, whereas 1.8 and 1.2 μg/ml (0.064 and 0.043 μM) for that from 293/2c cells (Fig. 3 and data not shown). Time course analysis of apoA-I-mediated lipid release supported the idea of simultaneous release of phospholipid and cholesterol both from 293/2c cells and from 293/6c cells as cholesterol and phospholipid in the medium were well detectable as early as 1 h after apoA-I stimulation, and they increased linearly for 8 h (Fig. 4). Density gradient analysis of the medium demonstrated that both cholesterol and phospholipid were recovered in the fractions with a density peak at around 1.08 g/ml, indicating that HDL particles were generated from the ABCA7-GFP-expressing cells similarly to the ABCA1-GFP-expressing cells (Fig. 5).

Expression of ABCA7-GFP also induced cholesterol release in L929 cells. We reported elsewhere that L929 cells release phospholipid but not cholesterol by apoA-I (22). As shown in Fig. 6, parent L929 cells released only phospholipid to apoA-I even at high concentrations of apoA-I (*open symbols*). Stable expression of ABCA7-GFP protein in L929 cells caused substantial release of cholesterol together with enhancement of phospholipid release (*closed symbols*). The level of endogenous ABCA1 expression was not affected in L929 by transfection and expression of ABCA7 (Fig. 6*C*).

Both dBcAMP and PMA enhanced apoA-I-mediated lipid release and ABCA1-GFP protein level in 293/2c. In the presence of dBcAMP (300 μM) or PMA (320 nM) with apoA-I, significant increase was induced in cholesterol and phospholipid release from 293/2c cells (Fig. 7, *A* and *B*, *open columns*). The increase of the lipid release was correlated with elevation of ABCA1-GFP protein level evaluated by GFP-derived fluorescence intensity and Western blotting analysis (Figs. 7*C* and 8*A*, *lanes 2–7*). The effects of dBcAMP and PMA were slightly synergistic, although not additive, in the condition tested.

Previous works demonstrated protection of ABCA1 by apolipoprotein against thiol protease-mediated degradation (15, 22–24). ApoA-I increased protein levels of ABCA1 (Fig. 8*A*) and ABCA1-GFP (Fig. 7*C* and other data not shown) expressed in HEK293 cells, consistent with the previous similar experiments (23, 24). A thiol protease inhibitor, *N*-acetyl-Leu-Leu-norleucinal (ALLN), enhanced apoA-I-mediated lipid release and ABCA1-GFP protein level as well as the increase of GFP-derived fluorescence in 293/2c (Figs. 7, *A–C*, and 8*A*).

In contrast, treatment with dBcAMP did not affect apoA-I-mediated cholesterol or phospholipid release in 293/6c cells, and PMA decreased release of both lipids (Fig. 9, *A* and *B*, *shadowed columns*). On the other hand, apoA-I and ALLN caused the increase of ABCA7-GFP in 293/6c, although the increase in cholesterol and phospholipid release was not statistically significant (Figs. 8*B*, 9, *A* and *B*, *hatched columns*). ABCA7-GFP protein level was not significantly affected either by dBcAMP, whereas it was slightly up-regulated by PMA (Figs. 8*B* and 9*C*). None of the compounds tested influenced non-specific cholesterol release to 2-hydroxypropyl-β-cyclodextrin from 293/2c or 293/6c (data not shown). These effects of dBcAMP, PMA, and apoA-I were similar in HEK293 cells transiently or stably expressing ABCA7 (Fig. 8*B*, *lanes 1–4*, and data not shown)

Induction of ABCA1 and ABCA7 by dBcAMP or PMA was further investigated in 293/2c and 293/6c cells, respectively, by monitoring their GFP-derived fluorescence (Fig. 10). In 293/2c cells, PMA induced the transient increase of ABCA1-GFP fluorescence, as the increase was evident after 2 h, reaching a peak at 8–12 h (Fig. 10*A*). dBcAMP-induced fluorescence increase was rather continuous at least up to 30 h after a time lag of 4–6 h. The effect of these two compounds were additive. Continuous monitoring of cellular fluorescence in the fluorescence-free medium yielded similar results (data not shown). Being consistent with these results, the apoA-I-mediated lipid release from 293/2c for 4 h was not affected by dBcAMP but was enhanced by PMA (data not shown). In 293/6c cells, fluorescence level was changed neither by dBcAMP nor by PMA (Fig. 10*B*). Fluorescent levels were increased in parallel with lipid release in 293/2c cells when treated with ALLN, whereas the increase was slight and unsustainable in 293/6c (data not shown).

G66976, a PKC inhibitor (25), reversed all of the changes caused by PMA. In 293/2c cells, the enhancement of apoA-I-mediated lipid release and ABCA1-GFP induction was inhibited (Figs. 8*A* and 11, *A–C*). In 293/6c cells, suppression of the apoA-I-mediated lipid release by PMA was recovered (Fig. 11, *D* and *E*), whereas the ABCA7-GFP level was reduced to the control level (Figs. 8*B* and 11*F*). G66976 alone had no signifi-

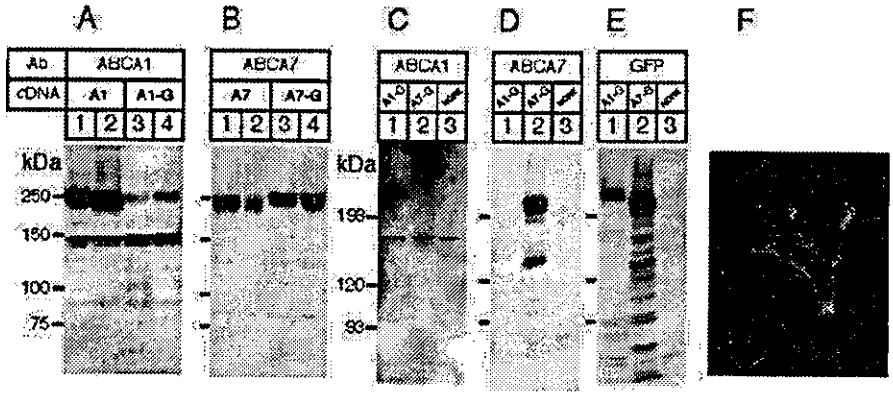

FIG. 2. **Detection of ABCA1- and ABCA7-containing proteins in HEK293 cells.** *A* and *B*, Western blotting analysis of transiently expressed proteins. Parent HEK293 cells were cultured and transfected with ABCA1/pcDNA3.1/Hygro (*A*, *lanes 1* and *2*), ABCA1-GFP/pcDNA3.1 (*A*, *lanes 3* and *4*), ABCA7/pCMV6c (*B*, *lanes 1* and *2*), and ABCA7-GFP/pcDNA3.1 (*B*, *lanes 3* and *4*) as in Fig. 1. Cells were collected after 30 h for crude membrane preparation. Membrane proteins (60 μg for *A*, *lanes 1* and *2* and *B*; 40 μg for *A*, *lanes 3* and *4*) were separated on a 5.5% SDS-polyacrylamide gel, transferred onto Immobilon (Millpore), and analyzed by using rabbit anti-ABCA1 antiserum (*A*) and rat polyclonal anti-ABCA7 antibody (*Ab*) (*B*). *C–E*, Western blotting analysis of 293/2c, 293/6c, and parent HEK293 cells. Cells were cultured in 10% FCS-DF and processed to prepare crude membrane fraction. 20 μg of membrane proteins were separated as above and analyzed by using rabbit anti-ABCA1 antiserum (*C*), rat polyclonal anti-ABCA7 antibody (*D*), and mouse monoclonal anti-GFP antibody (*E*). *Lane 1*, 293/2c; *lane 2*, 293/6c; *lane 3*, parent HEK293. *F*, subcellular localization of ABCA7-GFP protein. The 293/6c cells were cultured on a 35-mm glass-dish (Iwaki). Fluorescence images of the GFP were obtained as described under "Experimental Procedures."

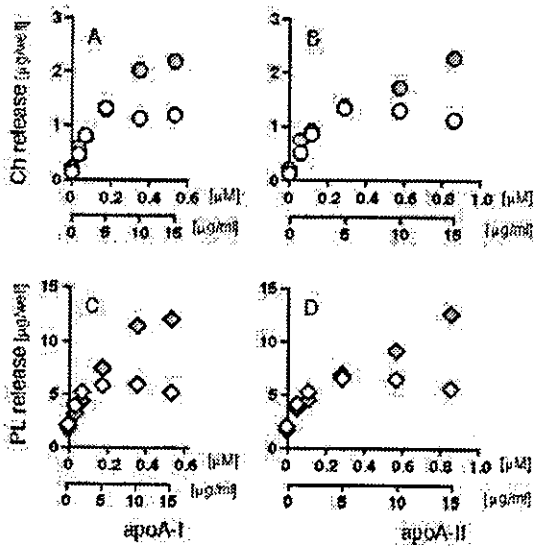

FIG. 3. **Release of cholesterol (*Ch*, *A* and *B*) and choline-phospholipids (*PL*, *C* and *D*) from HEK293 cells mediated by apoA-I (*A* and *C*) and apoA-II (*B* and *D*).** Cells were subcultured in 6-well trays at a density of 1.0×10^6 cell/well and incubated for 48 h. After washing with buffer H, cells were incubated in 1 ml/well of 0.02% BSA-DF containing apolipoproteins at the concentrations indicated. Concentrations of cholesterol and choline-phospholipid in the medium from 293/2c (*open symbols*) and 293/6c (*shadowed symbols*) cells were determined after 16 h. Results shown are the average and variation for two samples. *Error bars* are not shown when found to lie within the symbols. The amounts of total cellular cholesterol and choline-phospholipids in the cells at the starting time of apoA-I incubation were 9.2 ± 0.6 and 40.4 ± 2.4 μg/well in 293/2c cells and 9.2 ± 0.5 and 36.2 ± 2.3 μg/well in 293/6c cells, respectively (average ± S.D. for eight samples).

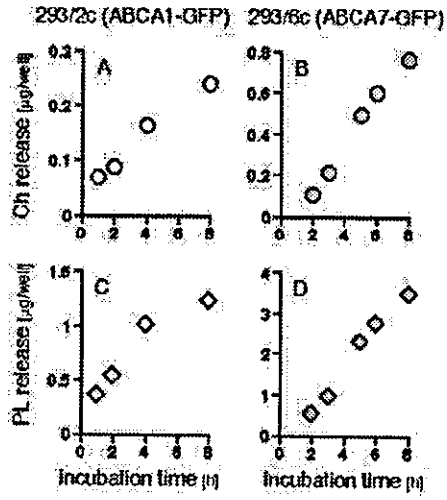

FIG. 4. **Simultaneous and time-dependent increase in apoA-I-mediated release of cholesterol (*Ch*) and choline-phospholipid (*PL*) from 293/2c and 293/6c cells.** 293/2c (*A* and *B*) and 293/6c (*C* and *D*) cells were cultured for 48 h as in Fig. 3. After washing with buffer H, cells were incubated in 0.02% BSA-DF containing 10 μg/ml apoA-I. After the incubation time indicated, cholesterol (*A* and *C*) and choline-phospholipid (*B* and *D*) content in the medium was measured. Results shown are representative from two independent sets of experiments after subtraction of the background. Each data point is from a single sample for 293/2c and from two samples for 293/6c.

cant effects on apoA-I-mediated lipid release from 293/2c or 293/6c (Fig. 11).

DISCUSSION

The function and its regulation of human ABCA7 was studied by using its expressing system of in HEK293 cells. ABCA7 exhibited a function for generation of cholesterol-containing HDL upon the interaction with apoA-I and apoA-II so much as human ABCA1 does. Response of ABCA7 to protein kinase modulators, dBcAMP and PMA, was somewhat different from ABCA1. ABCA1 protein level was increased by either reagent, and its function for mediating the apoA-I-mediated lipid release was increased in parallel, whereas ABCA7 and its activity were not increased by dBcAMP. Interestingly, ABCA7 was slightly increased by PMA, but its activity for mediating lipid release by apolipoprotein was rather suppressed. All of the effects of PMA were reversed by PKC-specific inhibitor G56976, indicating that they are mediated by PKC.

Deficiency of ABCA1 causes loss of plasma HDL, as demonstrated in the patients with Tangier disease (5–9) and in ABCA1 knockout mice (26, 27), to indicate that there is no significant compensatory backup system for supply of plasma HDL. At the cellular level, however, ABCG1 may function as a

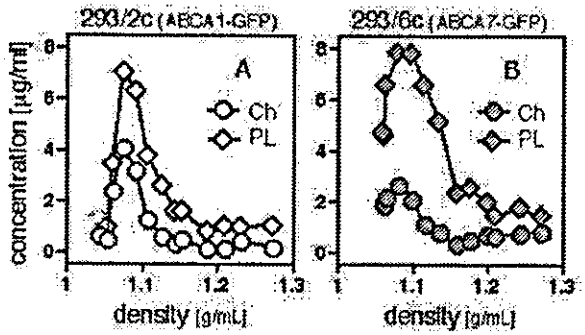

FIG. 5. **Density gradient analysis of lipids released from 293/2c (*A*) and 293/6c (*B*) cells.** Cells were cultured and stimulated with 10 μg/ml apoA-I. Medium was processed as described under "Experimental Procedures."

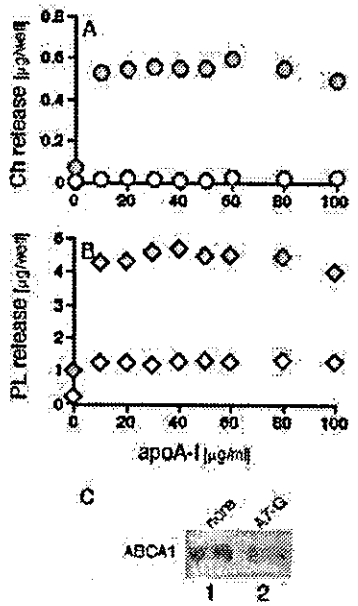

FIG. 6. **Effect of ABCA7 expression in L929 cells.** *A* and *B*, release of cholesterol (*Ch*, *A*) and choline-phospholipids (*PL*, *B*) mediated by apoA-I from parent L929 cells (*open symbols*) and L929 cells stably expressing ABCA7-GFP protein (mixture of several clones)(*shadowed symbols*). Cells were subcultured in 6-well trays at a density of 5.0×10^5 cell/well and processed as in Fig. 3. Results shown are the average and variation for two samples. *Error bars* are not shown when found to lie within the symbols. The amounts of total cellular cholesterol and choline-phospholipids in the cells at the starting time of apoA-I-incubation were 12.1 ± 0.5 and 43.3 ± 0.4 μg/well in parent L929 cells and 10.3 ± 0.2 and 68.3 ± 3.7 μg/well in L929 cells stably expressing ABCA7-GFP, respectively (average ± S.D. for six samples). *C*, ABCA1 protein level in L929 cells. Parent L929 cells (*lane 1*) and ABCA7-GFP-expressing L929 cells (*lane 9*) were cultured and processed as described in the legend for Fig. 2. Membrane protein (100 μg) was analyzed by Western blotting for each cell line cells.

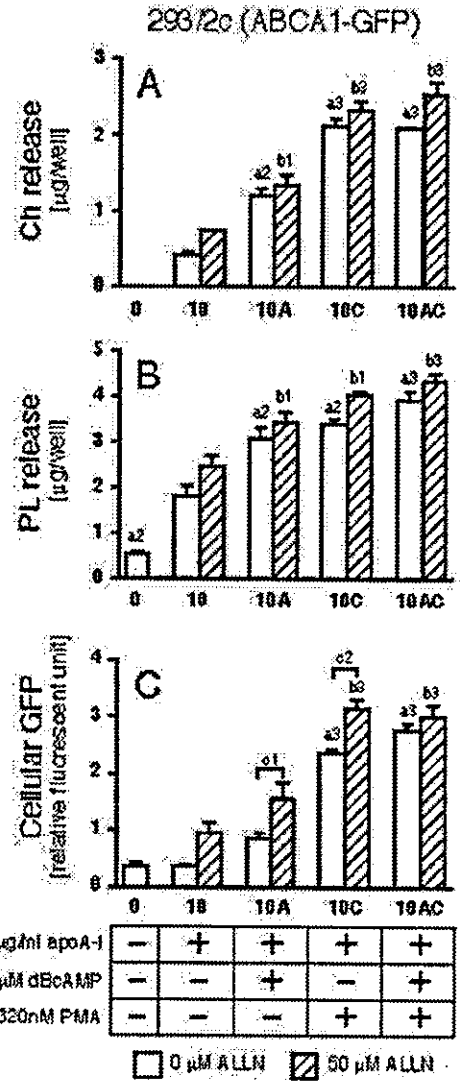

FIG. 7. **Effects of dBcAMP, PMA and ALLN on apoA-I-mediated lipid release and ABCA1-GFP protein level in 293/2c cells.** 293/2c cells were cultured for 48 h as described for Fig. 3. The cells were then washed with buffer H, cultured in 0.02% BSA-DF without (*open columns*) or with (*hatched columns*) 50 μM ALLN and reagents indicated. Medium was collected after a 9-h incubation for lipid analysis. Remaining cells were washed with buffer H, and GFP-derived fluorescence was measured. Results represent the average and variation for two samples of the lipid release and means ± S.D. for three samples of cellular GFP, respectively. *a1*, *a2*, and *a3*, $p < 0.05$, 0.01, and 0.001 *versus* group 10 without ALLN; *b1*, *b2*, and *b3*, $p < 0.05$, 0.01, and 0.001 *versus* group 10 with ALLN; *c1*, *c2*, *c3*, $p < 0.05$, 0.01, and 0.001 among the groups indicated. *Ch*, cholesterol; *PL*, phospholipid.

regulator of lipid transport in lipid-laden macrophages, although its *in vivo* function is not defined. Suppression of ABCG1 expression with the ABCG1-specific antisense oligonucleotide caused 32 and 25% reduction of the release of cholesterol and phospholipid, respectively, whereas ABCA1 expression was not down-regulated (10). It was also shown that levels of ABCG1 mRNA in non-cholesterol-laden macrophages from two patients with Tangier disease were significantly greater than controls, although the function of ABCG1 was not examined (28).

We demonstrated that human ABCA7 mediates the apolipoprotein-dependent cellular lipid release and consequent assembly of new HDL in a very similar manner as human ABCA1 *in vitro*. Our experimental protocol fulfills the condition to observe the effect of ABCA7 in the absence of ABCA1 so that this is an isolated function of this protein, at least *in vitro*. The results from L929 cells expressing ABCA7-GFP in addition to their endogenous ABCA1 (Fig. 6) suggested that the effects of ABCA1 and ABCA7 in apolipoprotein-mediated HDL generation may be synergistic. However, it is not clear whether ABCA1 and ABCA7 generate HDL particles independently, cholesterol-deficient HDLs and cholesterol-containing HDLs in this case. The reaction mechanisms of ABCA1 and ABC7 should be further investigated, including their difference, cross-talk, and physiological relevance.

In HEK293 cells, release of lipid may reach maximum at a

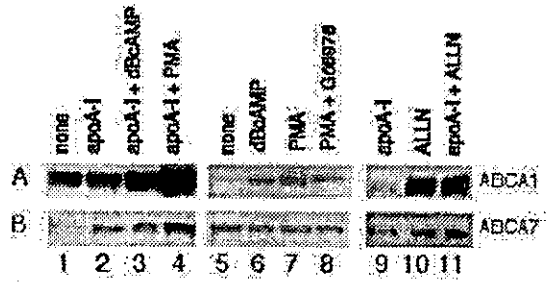

FIG. 8. **Effects of apoA-I, dBcAMP, PMA, G66976, and ALLN on ABCA1 and ABCA7 protein levels.** HEK293 cells stably expressing ABCA1 (mixture of several clones, *A, lanes 1–4*), ABCA1-GFP (clone 293/2c, *A, lanes 5–11*), ABCA7 (mixture of several clones, *B, lanes 1–4*), and ABCA7-GFP (clone 293/6c, *B, lanes 5–11*) were cultured as described under "Experimental Procedures." After a 48-h incubation, cells were washed with buffer H, and then 0.02% BSA-DF medium containing the compounds indicated was added. The final concentration of apoA-I, dBcAMP, PMA, G66976 and ALLN was 10 μg/ml, 300 μM, 320 nM, 10 μM, and 50 μM, respectively. Cells were cultured for another 16 h and collected for membrane preparation. ABCA1 (*A*) and ABCA7 (*B*) protein were detected by Western blotting.

slightly lower concentration of apoA-I and apoA-II (2–5 μg/ml) when ABCA1 was expressed than in the cells expressing ABCA7 (maximum at 20–30 μg/ml apoA-I) (Figs. 1 and 3). The results with ABCA1 were apparently consistent with previous findings with RAW264 where ABCA1 was strongly induced by dBcAMP, as shown by oligonucleotide array analysis that the mRNA level was increased 10-fold, whereas ABCA7 mRNA level was low and not affected (18). A similar dose-dependent curve was observed with human fibroblast WI-38 cells in which ABCA1 was found (22) but not ABCA7 (data not shown). Further investigation is required for understanding the underlying mechanism for this apparent difference in kinetic profiles of the HDL assembly reactions between ABCA1 and ABCA7.

HEK293 cells transiently expressing mouse ABCA7 released phospholipids but not cholesterol by apoA-I, even in the presence of scavenger receptor-BI or loading of extra cholesterol mass (13). As HEK293 cells transiently expressing mouse ABCA1 were able to generate cholesterol-containing HDL (13, 29), the difference may be between human ABCA7 and mouse ABCA7.

The effect of dBcAMP on ABCA1-GFP level in 293/2c is consistent with the previous reports, an increase of ABCA1 mRNA in human fibroblasts (22), RAW264 cells (18, 30), and macrophages (31) by cAMP analogues. It has been reported that there is a cAMP-responsive element in the ABCA1 promoter (32). However, neither ABCA1 protein (Fig. 1 and data not shown) nor apoA-I-mediated HDL generation (Table I) was detected in parent HEK293 cells even after the dBcAMP treatment so that it is unlikely that the enhancement of the ABCA1 activity by dBcAMP in 293/2c is carried out by this cAMP-responsive element of the endogenous ABCA1 gene. In fact, no immunoreactive band was detected in 293/2c at the position of lower molecular weight than ABCA1-GFP even after the cAMP treatment. The promoters of the transfected cDNAs were common (immediate early promoter of cytomegalovirus) for ABCA1-GFP and ABCA7-GFP and dBcAMP did not increase ABCA7-GFP so that the effect of cAMP in this case is likely to be on the post-transcriptional modulation.

ABCA1 protein expressed in *Xenopus* oocytes was phosphorylated by PKA in a cell-free system (33), and its activity as an anion transporter was enhanced after short term treatment with PKA activators (33). Also, 8-bromo-cAMP promoted phosphorylation of ABCA1 by a 1-h incubation in normal human fibroblasts and increased the apoA-I-mediated release of cholesterol and phospholipid without changing its mRNA or protein level (34). A more recent report indicated that phosphorylation by PKA at a specific site of ABCA1 is constitutive but important for the apoA-I-mediated phospholipid release (35). Our results seem rather consistent with the cellular conditions similar to *Xenopus* oocytes (33) or fibroblasts (34). Regulation of ABCA7 by PKA may then be different in this regard.

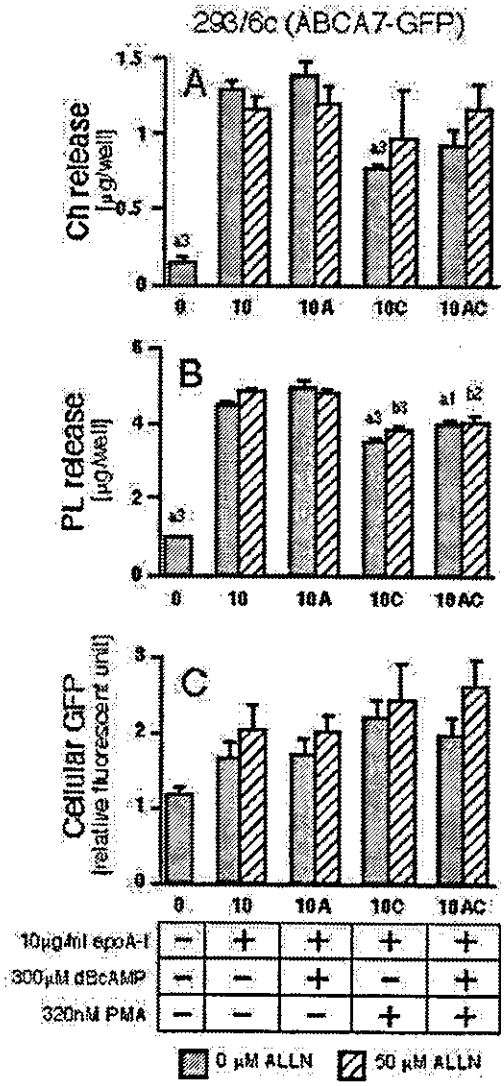

FIG. 9. **Effects of dBcAMP, PMA and ALLN on apoA-I-mediated lipid release and ABCA7-GFP protein level in 293/6c cells.** 293/6c cells were processed in the same way as in Fig. 7. Results represent the average and variation for two samples. Legends are same as in Fig. 7. *Ch*, cholesterol; *PL*, phospholipid.

The effect of the PKC activator, PMA, also differentiated the response of ABCA1 and ABCA7. ABCA1-GFP in 293/2c was increased by PMA, and the activity also seemed increased in parallel (Fig. 7). In contrast, PMA decreased the apoA-I-mediated lipid release from 293/6c expressing ABCA7-GFP (Fig. 9, *A* and *B*), whereas it did not cause significant reduction of the GFP-derived fluorescence (Figs. 9*C* and 11*F*). These effects of PMA were all reversed by Gö6976, an inhibitor of Ca^{2+}-dependent isoform(s) of PKC (25). The data indicate that specific activities of ABCA7 can be modulated by PKC.

Turnover of ABC transporters has not been fully understood. We recently reported that degradation of ABCA1 is protected by apolipoproteins from the degradation by thiol protease, most likely calpain (15, 24). ALLN was effective to increase ABCA1

protein expressed in HEK293 cells, indicating that ABCA1 protein expressed by the exogenously transfected cDNA is metabolized by the similar mechanism, being consistent with other reports (23, 24). The results with the transfected ABCA7 were largely similar to those with ABCA1, showing that the metabolic pathway of these proteins are common.

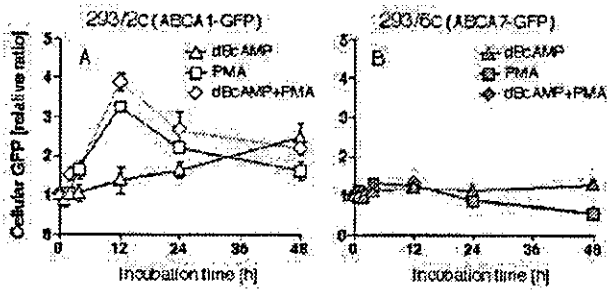

FIG. 10. **Time course analysis in GFP-derived fluorescence in 293/2c (*A*) and 293/6c (*B*).** The clone cells were subcultured into 12-well trays 5.0×10^5 cells/well and cultured for 64 h in 10% FCS-DF. Reagents indicated were added into the wells at the time 16, 40, 52, 60, 62, and 63 h after subculture. The final concentrations of dBcAMP and PMA were 300 μM and 320 nM, respectively. At 64 h of the incubation after subculture, medium was removed, cells were washed with buffer H, and cellular fluorescence was measured. Data are relative to control cells that were maintained in 10% FCS-DF throughout the experiment. In this experimental condition, a 48-h incubation with dBcAMP did not affect cell growth, whereas PMA suppressed it by about 15%. These effects were same for parent HEK293 cells, 293/2c cells, and 293/6c cells. Results represent means ± S.D. for three samples.

The physiological relevance of the activity of ABCA7 reported in this study should be further investigated. ABCA1 distribution is ubiquitous (33), and its dysfunction results in the loss of plasma HDL (5–9), whereas tissue distribution of ABCA7 is reportedly restricted to myelo-lymphatic tissues in human (11) and mouse (36) or preferentially in platelets in rat (21) and mouse (13). Therefore, the ABCA7-mediated lipid release may not contribute significantly to a source of plasma HDL. However, it may still play an important role in cellular cholesterol homeostasis in particular tissues including macrophages. In Tangier disease, accumulation of cholesteryl ester is found in foamy histiocytes in the reiticuloendotherial system, fibroblasts of the cornea, melanocytes, Schwann cells, neurons, and non-vascular-muscle cells (37). It is interesting to point out that Tangier disease patients appear to be only at moderately increased cardiovascular risk despite the almost complete loss of plasma HDL and considerable cholesterol ester accumulation in resident macrophages of many tissues (37). ABCA1 knockout mice showed tissue distribution of lipid deposition identical to Tangier disease and no abnormalities in aorta even in aged mice (38). These findings may suggest that there is a protecting system against the development of atherosclerosis even in the absence of ABCA1, and the ABCA7-mediated lipid

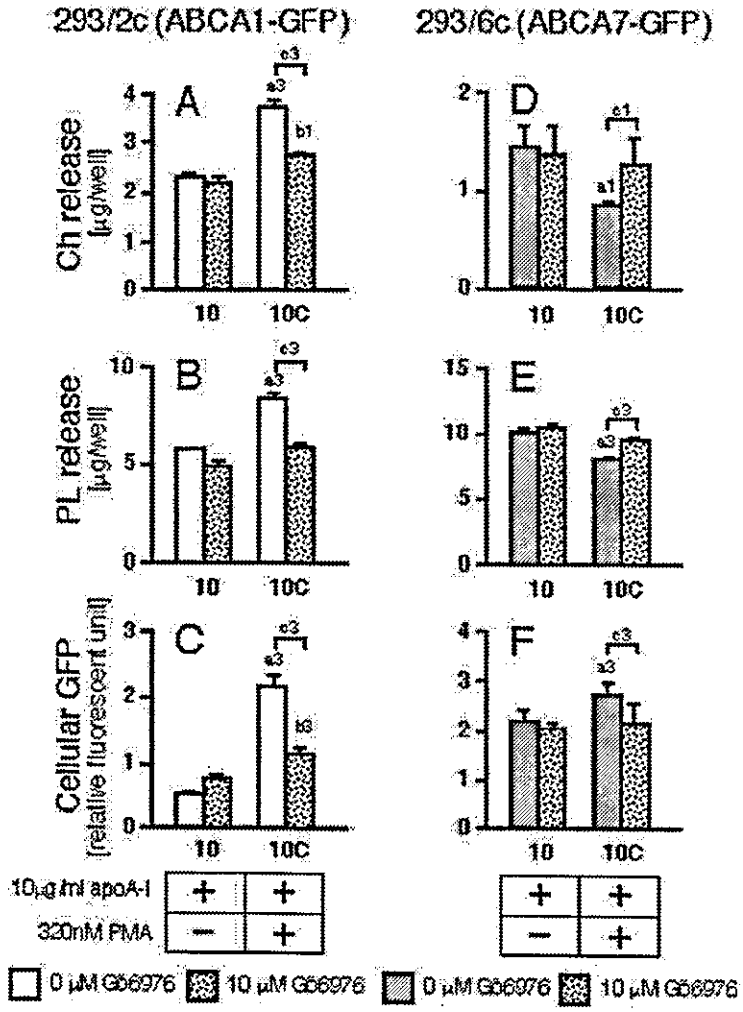

FIG. 11. **Effects of Gö6976 on the apoA-I-mediated lipid release and the GFP protein level for 293/2c (*A–C*) and 293/6c (*D–F*).** The cells were cultured for 48 h as described in the legend for Fig. 3. The cells were then washed with buffer H, cultured in 0.02% BSA-DF without (*open columns*) or with (*hatched columns*) 10 μM Gö6976 and substances indicated. Medium was collected after an 18-h incubation for lipid analysis. Remaining cells were processed as in Fig. 7. Results represent means ± S.D. for three and six samples in lipid release and cellular GFP, respectively. *a1* and *a3*, $p < 0.05$ and 0.001 *versus* group 10 without Gö6976; *b1* and *b3*, $p < 0.05$ and 0.001 *versus* group 10 with Gö6976; *c1* and *c3*, $p < 0.05$ and 0.001 between the groups indicated. *Ch*, cholesterol; *PL*, phospholipid.

release from macrophages in the vascular wall is one of the candidates. In fact, ABCA7 protein is detected in peripheral blood monocytes after *in vitro* differentiation into macrophages followed by acetylated LDL loading (11). Specific roles of this protein should be examined, especially in atherosclerotic tissues.

Further study of the mechanism by which ABCA7 mediates the release of cholesterol and generation of HDL should provide us with important information for intracellular trafficking and homeostasis of cholesterol by comparing it with ABCA1. It would also lead us to novel strategies for treatment of atherosclerosis. Controlled induction of ABCA7 in certain specific organs, tissues, or cells, such as macrophages and lymphomyeloid cells, would be efficient to remove cholesterol from peripheral tissues to prevent atherosclerosis, by itself or in coordination with ABCA1.

Acknowledgments—We thank Kyowa Hakko Co. Ltd. for generating anti-ABCA7 polyclonal antibody and Tatsuya Kishimoto at ASWELL Inc. for providing reagents for lysoPC assay. We are also grateful to Michiyo Asai for preparation of apolipoproteins. Naotaka Ninaguwa, Kaoru Kuzuya, Hiroyuki Imafuji, and Akemi Miyata, students of Nagoya City University Medical School, contributed to the initial stage of the project.

REFERENCES

1. Hara, H., and Yokoyama, S. (1991) *J. Biol. Chem.* **260,** 3080–3086
2. Yokoyama, S. (2000) *Biochim. Biophys. Acta* **1529,** 231–244
3. Francis, G. A., Knopp, R. H., and Oram, J. F. (1995) *J. Clin. Invest.* **96,** 78–87
4. Remaley, A. T., Schumacher, U. K., Stonik, J. A., Farsi, B. D., Nazih, H. B., and Brewer, H. B. J. (1997) *Arterioscler. Thromb. Vasc. Biol.* **17,** 1813–1821
5. Bodzioch, M., Orso, E., Klucken, J., Langmann, T., Böttcher, A., Diederich, W., Drobnik, W., Barlage, S., Büchler, C., Porsch-Özcürümez, M., Kaminski, W. E., Hahmann, H. W., Oette, K., Rothe, G., Aslanidis, C., Lackner, K. J., and Schmitz, G. (1999) *Nat. Genet.* **22,** 347–351
6. Brooks-Wilson, A., Marcil, M., Clee, S. M., Zhang, L.-H., Roomp, K., van Dam, M., Yu, L., Brewer, C., Collins, J. A., Molhuizen, H. O. F., Loubser, O., Ouelette, B. F. F., Fichter, K., Ashbourne-Excoffon, K. J. D., Sensen, C. W., Scherer, S., Mott, S., Denis, M., Martindale, D., Frohlich, J., Morgan, K., Koop, B., Pimstone, S., Kastelein, J. J. P., Genest, J., Jr., and Hayden, M. R. (1999) *Nat. Genet.* **22,** 336–345
7. Marcil, M., Brooks-Wilson, A., Clee, S. M., Roomp, K., Zhang, L. H., Yu, L., Collins, J. A., van Dam, M., Molhuizen, H. O., Loubster, O., Ouellette, B. F., Sensen, C. W., Fichter, K., Mott, S., Denis, M., Boucher, B., Pimstone, S., Genest, J., Jr., Kastelein, J. J., and Hayden, M. R. (1999) *Lancet* **354,** 1341–1346
8. Remaley, A. T., Rust, S., Rosier, M., Knapper, C., Naudin, L., Broccardo, C., Peterson, K. M., Koch, C., Arnould, I., Prades, C., Duverger, N., Funke, H., Assman, G., Dinger, M., Dean, M., Chimini, G., Santamarina-Fojo, S., Fredrickson, D. S., Denefle, P., and Brewer, H. B., Jr. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 12685–12690
9. Rust, S., Rosier, M., Funke, H., Real, J., Amoura, Z., Piette, J.-C., Deleuze, J.-F., Brewer, H. B., Duverger, N., Denefle, P., and Assmann, G. (1999) *Nat. Genet.* **22,** 352–355
10. Klucken, J., Büchler, C., Orsó, E., Kaminski, W. E., Porsch-Özcürümez, M., Liebisch, G., Kapinsky, M., Diederich, W., Drobnik, W., Dean, M., Allikmets, R., and Schmitz, G. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 817–822
11. Kaminski, W. E., Piehler, A., and Schmitz, G. (2000) *Biochem. Biophys. Res. Commun.* **278,** 782–789
12. Tanaka, A. R., Ikeda, Y., Abe-Dohmae, S., Arakawa, R., Sadanami, K., Kidera, A., Nakagawa, S., Nagase, T., Aoki, R., Kioka, N., Amachi, T., Yokoyama, S., and Ueda, K. (2001) *Biochem. Biophys. Res. Commun.* **283,** 1019–1025
13. Wang, N., Lan, D., Gerbod-Giannone, M., Linsel-Nitschke, P., Jehle, A. W., Chen, W., Martinez, L. O., and Tall, A. R. (2003) *J. Biol. Chem.* **278,** 42906–42912
14. Kojima, K., Abe-Dohmae, S., Arakawa, R., Murakami, I., Suzumori, K., and Yokoyama, S. (2001) *Biochim. Biophys. Acta* **1532,** 173–184
15. Arakawa, R., and Yokoyama, S. (2002) *J. Biol. Chem.* **277,** 22426–22429
16. Yokoyama, S., Tajima, S., and Yamamoto, A. (1982) *J. Biochem. (Tokyo)* **91,** 1267–1272
17. Tajima, S., Yokoyama, S., and Yamamoto, A. (1983) *J. Biol. Chem.* **258,** 10073–10082
18. Abe-Dohmae, S., Suzuki, S., Wada, Y., Aburatani, H., Vance, D. E., and Yokoyama, S. (2000) *Biochemistry* **39,** 11092–11099
19. Kishimoto, T., Soda, Y., Matsuyama, Y., and Mizuno, K. (2002) *Clin. Biochem.* **35,** 411–416
20. Tanaka, A. R., Abe-Dohmae, S., Ohnishi, T., Aoki, R., Morinaga, G., Okuhira, K., Ikeda, Y., Kano, F., Matsuo, M., Kioka, N., Amachi, T., Murata, M., Yokoyama, S., and Ueda, K. (2003) *J. Biol. Chem.* **278,** 8815–8819
21. Sasaki, M., Shoji, A., Kubo, Y., Nada, S., and Yamaguchi, A. (2003) *Biochem. Biophys. Res. Commun.* **304,** 777–782
22. Yamauchi, Y., Abe-Dohmae, S., and Yokoyama, S. (2002) *Biochim. Biophys. Acta* **1585,** 1–10
23. Wang, N., Chen, W., Linsel-Nitschke, P., Martinez, L. O., Agerholm-Larsen, B., Silver, D. L., and Tall, A. R. (2003) *J. Clin. Invest.* **111,** 99–107
24. Yamauchi, Y., Hayashi, M., Abe-Dohmae, S., and Yokoyama, S. (2003) *J. Biol. Chem.* **278,** 47890–47897
25. Martiny-Baron, G., Kazanietz, M. G., Mischak, H., Blumberg, P. M., Kochs, G., Hug, H., Marme, D., and Schachtele, C. (1993) *J. Biol. Chem.* **268,** 9194–9197
26. Orso, E., Broccardo, C., Kaminski, W. E., Böttcher, A., Liebisch, G., Drobnik, W., Götz, A., Chambenoit, O., Diederich, W., Langmann, T., Spruss, T., Luciani, M.-F., Rothe, G., Lackner, K. J., Chimini, G., and Schmitz, G. (2000) *Nat. Genet.* **24,** 192–196
27. McNeish, J., Aiello, R. J., Guyot, D., Turi, T., Gabel, C., Aldinger, C., Hoppe, K. L., Roach, M. L., Royer, L. J., de Wet, J., Broccardo, C., Chimini, G., and Francone, O. L. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 4245–4250
28. Lorkowski, S., Kratz, M., Wenner, C., Schmidt, R., Weitkamp, B., Fobker, M., Reinhardt, J., Rauterberg, J., Galinski, E. A., and Cullen, P. (2001) *Biochem. Biophys. Res. Commun.* **283,** 821–830
29. Wang, N., Silver, D. L., Thiele, C., and Tall, A. R. (2001) *J. Biol. Chem.* **276,** 23742–23747
30. Smith, J. D., Miyata, M., Ginsberg, M., Grigaux, C., Shmookler, E., and Plump, A. S. (1996) *J. Biol. Chem.* **271,** 30647–30655
31. Oram, J. F., Lawn, R. M., Garvin, M. R., and Wade, D. P. (2000) *J. Biol. Chem.* **34508–34511**
32. Santamarina-Fojo, S., Peterson, K., Knapper, C., Qiu, Y., Freeman, L., Cheng, J. F., Osorio, J., Remaley, A., Yang, X. P., Haudenschild, C., Prades, C., Chimini, G., Blackmon, E., Francois, T., Duverger, N., Rubin, E. M., Rosier, M., Denefle, P., Fredrickson, D. S., and Brewer, H. B., Jr. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7987–7992
33. Becq, F., Hamon, Y., Bajetto, A., Gola, M., Verrier, B., and Chimini, G. (1997) *J. Biol. Chem.* **272,** 2695–2699
34. Haidar, B., Denis, M., Krimbou, L., Marcil, M., and Genest, J., Jr. (2002) *J. Lipid Res.* **43,** 2087–2094
35. See, R. H., Caday-Malcolm, R. A., Singaraja, R. R., Zhou, S., Silverston, A., Huber, M. T., Moran, J., James, E. R., Janoo, R., Savill, J. M., Rigot, V., Zhang, L. H., Wang, M., Chimini, G., Wellington, C. L., Tafuri, S. R., and Hayden, M. R. (2002) *J. Biol. Chem.* **277,** 41835–41842
36. Broccardo, C., Osorio, J., Luciani, M. F., Schriml, L. M., Prades, C., Shulenin, S., Arnould, I., Naudin, L., Lafargue, C., Rosier, M., Jordan, B., Mattei, M. G., Dean, M., Denefle, P., and Chimini, G. (2001) *Cytogenet. Cell Genet.* **92,** 264–270
37. Assmann, G., von Eckardstein, A., and Brewer, H. B., Jr. (2001) in *The Metabolic and Molecular Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. C., Valle, D., and Sly, W. S., eds) 8th Ed., pp. 2937–2960, McGraw-Hill, New York
38. Christiansen-Weber, T. A., Voland, J. R., Wu, Y., Ngo, K., Roland, B. L., Nguyen, S., Peterson, P. A., and Fung-Leung, W. P. (2000) *Am. J. Pathol.* **157,** 1017–1029

FULL PAPER *Biochemistry*

Effect of PCB-126 on Intracellular Accumulation and Transepithelial Transport of Vinblastine in LLC-PK1 and Its Transformant Cells Expressing Human P-Glycoprotein

Shigemi SASAWATARI[1], Minae TOKI[1], Takebumi HORIE[1], Yuki NAKANO[1], Teruo IKEDA[2,3], Kazumitsu UEDA[4] and Hiroshi FUJISE[1,2]*

[1]*Laboratory of Pathobiochemistry, School of Veterinary Medicine,* [2]*High-Tech Research Center, Research Institute of Biosciences, and* [3]*Laboratory of Microbiology I, Azabu University, Sagamihara, Kanagawa 229–8501 and* [4]*Laboratory of Cellular Biochemistry, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606–8502, Japan*

(Received 5 November 2003/Accepted 19 April 2004)

ABSTRACT. The effects of 3, 3', 4, 4', 5-pentachlorobiphenyl (PCB-126), which is the most toxic congener of coplanar polychlorinated biphenyls (Co-PCBs), on intracellular accumulation and transepithelial transport of vinblastine were examined in porcine kidney cells, LLC-PK1, and its transformant cells expressing human P-glycoprotein (LLC-MDR1). The accumulation decreased less than one-tenth in LLC-MDR1 compared to LLC-PK1. In both cells, the accumulation increased with the addition of PCB-126 and cyclosporine A (CYA), which are P-glycoprotein modulators, though the magnitudes were different in these two cell groups as well as for these two chemicals. Thus, PCB-126 might inhibit extrusion of vinblastine through the drug extrusion system as does CYA. In both the cells, there might be an endogenous drug extrusion system other than P-glycoprotein that was inhibited by CYA or PCB-126. The net basal-to-apical transepithelial transport of vinblastine increased 1.7-fold more in LLC-MDR1 than in LLC-PK1. By adding PCB-126 on the apical side, the transport was greatly decreased by -76% in the monolayer of both cells. By adding PCB-126 and CYA on the basal side in LLC-MDR1 monolayer, the transports increased -1.7-fold, so that PCB-126 might inhibit the extrusion of vinblastine on both the apical and basal sides. One of the causes to be considered for the adverse effects of Co-PCBs, in addition to the binding with an aryl hydrocarbon receptor, might be the modification of drug transport by its interaction with the drug transport system.

KEY WORDS: LLC-PK1, 3,3',4,4',5-pentachlorobiphenyl, P-glycoprotein, transepithelial transport, vinblastine.

J. Vet. Med. Sci. 66(9): 1079–1085, 2004

Coplanar polychlorinated biphenyls (Co-PCBs) are environmental pollutants, and soil, rivers and oceans have been widely degraded by them. Due to the food chain and bioaccumulation, these chemicals might have become highly accumulated in humans and animals [1, 3, 6, 12, 22]. It was considered that almost the toxic effects of Co-PCBs were initiated by binding with the aryl hydrocarbon receptor (AhR) [18, 24], and these chemicals induced estrogenic and antiestrogenic responses via the crosstalk between AhR and estrogen receptor [8, 31]. Thus, the toxicity of Co-PCBs has appeared in the form of endocrine disruptors and has been found to cause many adverse effects such as reproductive disorders, malformation and cancer [7, 16, 17, 20, 22]. In addition to such toxicities, Co-PCBs might affect some other parts of the animal body. During our survey to determine whether Co-PCBs were transported by a drug extrusion pump such as P-glycoprotein, we found that Co-PCBs seemed to inhibit the extrusion of other chemicals in human epidermoid carcinoma cells, such as KB-3, expressed with P-glycoprotein [2, 11].

P-glycoprotein transports lipophylic chemicals, and thus it plays an important role in transporting metabolites and extruding toxic chemicals from epithelial cells [4, 5, 14, 28]. There are also substrates of P-glycoprotein which are not transported, but are bound to P-glycoprotein and inhibit the transport of other chemicals [4, 25]. Therefore, there is a possibility that Co-PCBs are the substrates for the binding of P-glycoprotein, and inhibit drug extrusion in KB-3 cells. In our previous experiments, we measured drug accumulation in KB-3 cells [2, 11]. Thus, doubts may arise as to whether the values reflected inhibition of transport or competition of drug adsorption by lipophilic Co-PCBs, because lipophilic compounds were readily adsorbed on the plasma membrane in a non-specific fashion. Therefore, we employed both measurements for the intracellular accumulation and transepithelial transport in the present experiment. One of the present authors showed that P-glycoprotein existed at the apical membrane in epithelial cells, and mediated transepithelial transport in the epithelial monolayer cells [30], so that it is important to examine the effects of Co-PCBs on the epithelial monolayer.

3,3',4,4',5-pentachlorobiphenyl (PCB-126) is the most toxic among the congeners of Co-PCBs, and has been detected in human blood [7, 23]. Here we report the effect of PCB-126 on the accumulation and transepithelial transport of vinblastine in a porcine kidney epithelial cell line, LLC-PK1, and its transformant cells expressed with human P-glycoprotein. Vinblastine is among the anticancer drugs, which has been well examined as a substrate of P-glycoprotein for transport [15, 19, 29]. Cyclosporine A (CYA) was employed as a positive control for an inhibitor of P-glyco-

* CORRESPONDENCE TO: FUJISE, H., Laboratory of Pathobiochemistry, School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa 229–8501, Japan.

protein, which has been shown to compete with vinblastine for a binding site of P-glycoprotein [25]. The measurement of the effects of Co-PCBs on the intracellular accumulation of the drug might reflect the cell toxicity, and the examination of the effect on the transepithelial transport could reveal a modification of drug transport across the epithelium.

MATERIALS AND METHODS

Chemicals: PCB-126 was purchased from Kanto Kagaku (Tokyo, Japan). CYA was obtained from Wako (Osaka, Japan), and vinblastine was obtained from Sigma (St. Louis, MO, USA). [G-^{3}H]-vinblastine sulphate (470 GBq/mmol) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA), and [carboxy-^{14}C]-inulin (92.5 GBq/g) was purchased from American Radio Labeled Chemical (St. Louis, MO, USA).

Cells: Porcine kidney epithelial cells, LLC-PK1, and their transformant cells expressing human P-glycoprotein, LLC-GA5-COL150 (LLC-MDR1), were employed as reported previously [27]. LLC-PK1 was maintained in medium 199 supplemented with 10% fetal calf serum, and LLC-MDR1 was cultured in that medium containing 150 μM colchicine in an atmosphere of 5% CO_2 at 37°C. In LLC-PK1, the inhibition dose for 50% cell growth in four days (ID_{50}) in the medium with colchicine and vinblastine was 28 nM and 6.2 nM, respectively. These ID50s with colchicine and vinblastine were increased by 19- and 52-fold, respectively, in LLC-MDR1.

Accumulation of vinblastine: For a determination of the intracellular accumulation of vinblastine, a cover slip and 24-well multi-dish (Nalge Nunc International, Rochester, NY, USA) were used as reported previously [9, 10]. The cells were seeded at 5×10^6/well in the medium, and incubated in 5% CO_2 at 37°C. After 6 days of incubation, the medium was replaced with a fresh medium without colchicine, and the cells were incubated for 6 hr. Then the medium was replaced with 750 μl fresh medium containing 11 nM [^{3}H]-vinblastine (5.16 kBq/m*l*) with or without CYA or PCB-126. After incubation for 1, 2 and 3 hr, the cover slip was removed and the cells were washed 3 times with PBS and lysed with lysate buffer; their radioactivity was then measured with a liquid scintillation counter. The accumulation of vinblastine in the cells was expressed as pmol/mg protein.

Transepithelial transports: Transepithelial transports, both basal-to-apical and apical-to-basal, were measured in the cell monolayer on a bottom-filtered well (12 mm diameter, Transwell, 3402, Costar, Cambridge, MA, USA) as reported previously [27, 30]. The cells were seeded on the bottom-filtered well in the same manner as in the accumulation experiment. After a 6-day incubation, the medium on either the basal or apical side of the monolayers was replaced with 750 μl fresh medium containing 11 nM [^{3}H]-vinblastine (5.16 kBq/m*l*) with 43.2 μg/m*l* [^{14}C]-inulin (4.0 kBq/m*l*) with or without 1 μM CYA or PCB-126. An aliquot (25 μl) of the receiver side medium was collected within 3 hr, and its radioactivity was measured with a liquid scintillation counter. The transepithelial transport was indicated as pmol per well. The paracellular fluxes were monitored by measuring the appearance of inulin on the other side, which turned out to be less than 5% at 3 hr as reported earlier [26].

Statistical analysis: Following the *F*-test, Student's *t*-test was employed to examine the statistical significance of the accumulations and transepithelial transports of those with and without inhibitors.

RESULTS

Figure 1 shows the intracellular accumulation of vinblastine as a function of time in the medium with or without CYA and PCB-126 in LLC-PK1 (A), and ratios of the accumulations without and with these chemicals after a 3-hr incubation (B). The accumulations hyperbolically increased in all the mediums with or without CYA and PCB-126, though the accumulation was higher in the medium with these chemicals than without them. The accumulation was 4.14 pmol/mg protein in the medium without these chemicals after 3-hr incubation, and it was increased by 2- and 1.8-fold in the medium with CYA and PCB-126, respectively, but there was no difference between the accumulations with CYA and PCB-126. Therefore, the extrusion of vinblastine was decreased by adding these chemicals in LLC-PK1.

Figure 2 shows the intracellular accumulation of vinblastine as a function of time in the medium with or without CYA and PCB-126 in LLC-MDR1 (A), and ratios of the accumulations without and with those chemicals at a 3-hr incubation (B). The accumulation was decreased to 0.30 pmol/mg protein after a 3-hr incubation in the medium with-

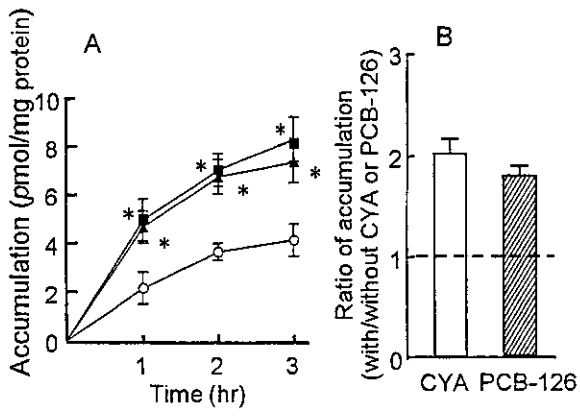

Fig. 1. Accumulations of vinblastine as a function of time in the medium without (open circles) and with 1 μM cyclosporine A (CYA, filled squares) or PCB-126 (filled triangles) in LLC-PK1 (A), and the ratio of the accumulation of vinblastine with and without CYA or PCB-126 (with/without CYA or PCB-126) in LLC-PK1 (B). Values are the means and SD of four experiments. *, Statistical significance between the accumulations with and without CYA or PCB-126 ($p<0.05$).

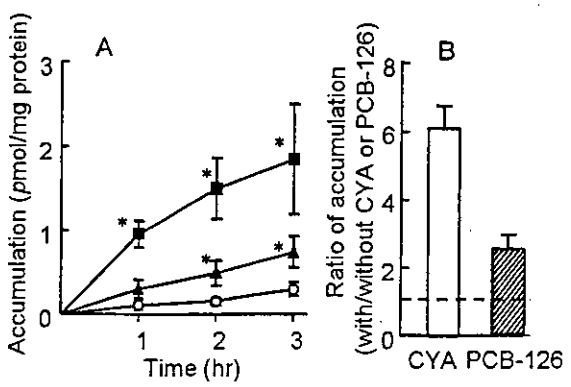

Fig. 2. Accumulations of vinblastine as a function of time in the medium without (open circles) and with 1 μM CYA (filled squares) or PCB-126 (filled triangles) in LLC-MDR1 (A), and ratio of the accumulation of vinblastine with and without CYA or PCB-126 (with/without CYA or PCB-126) in LLC-MDR1 (B). Values are the means and SD of four experiments. *, Statistical significance of the accumulations with and without CYA or PCB-126 ($p<0.05$).

out chemicals in LLC-MDR1: it was decreased by 93% compared to that in LLC-PK1 (Fig. 1A). In LLC-MDR1, the accumulations of vinblastine in the medium with CYA and PCB-126 were 6.1- and 2.5-fold higher, respectively, compared to that in the medium without these chemicals. Thus, PCB-126 inhibited the extrusion of vinblastine the same as CYA did, although the absolute magnitude of the inhibition with PCB-126 was lower than that with CYA.

Figure 3 shows the effect of CYA (A, B) and PCB-126 (C, D) at the apical (A, C) and basal sides (B, D) on the transepithelial transports of vinblastine in LLC-PK1, together with a comparison of ratios for the net basal-to-apical transport of without and with these chemicals (E). The basal-to-apical transport decreased and the apical-to-basal transport was increased by adding CYA at either the basal or apical side (A, B) in LLC-PK1. Thus, the net basal-to-apical transports with CYA on the apical and the basal sides decreased by 65 to 100% (E). By adding PCB-126 on the apical side, the basal-to-apical transport was greatly decreased by 85% at a 1-hr incubation, but the decrease was moderate at a 2- to 3-hr incubation, whereas the apical-to-basal transport was slightly increased at all time points (C). Thus, the net basal-to-apical transport was decreased by 76% at a 1-hr incubation in the medium with PCB-126 on the apical side (E), but the effects were more moderate later, and they were reduced by 36% in a 3-hr incubation compared to the control (E). With PCB-126 on the basal side, there were no significant changes in the net basal-to-apical transport since both the basal-to-apical and apical-to-basal transports increased to the same magnitude in LLC-PK1 all the time (D, E).

Figure 4 shows the effect of CYA (A, B) and PCB-126 (C, D) at the apical (A, C) and basal sides (B, D) on the transepithelial transports of vinblastine in the monolayer of LLC-MDR1, providing a comparison of the ratios for the net basal-to-apical transport without and with these chemicals (E). In the medium without CYA and PCB-126, the basal-to-apical transport increased and the apical-to-basal transport decreased in LLC-MDR1 (Fig. 4: open symbols) compared to LLC-PK1 (Fig. 3: open symbols). Thus, the net basal-to-apical transport increased 1.7-fold in the monolayer of LLC-MDR1 compared to that in LLC-PK1.

In LLC-MDR1, the basal-to-apical transport with CYA on the apical side was unchanged from that without the chemical, whereas there was a slight increase in the apical-to-basal transport with CYA on the apical side (A), but one that was too small to affect the net basal-to-apical transport. With CYA on the basal side, the basal-to-apical transport increased significantly, but the increase in the apical-to-basal transport was very small (B). Thus, the net basal-to-apical transport increased by around 1.4-fold when CYA was added on the basal side in LLC-MDR1 (E). With PCB-126 on the apical side, the basal-to-apical transport was greatly reduced and the apical-to-basal transport was unchanged at a 1-hr incubation, so the net basal-to-apical transport decreased by 55% compared to that without PCB-126 (C, E), but the basal-to-apical transports were unchanged in 2- to 3-hr incubation. Thus, no difference was found for the net transepithelial transports when using the medium with and without PCB-126 at the later time points. With PCB-126 on the basal side, the basal-to-apical transport was increased, but the apical-to-basal transport always remained unchanged (D). Thus, the net basal-to-apical transport increased to 1.7-fold when PCB-126 was added to the basal side in LLC-MDR1, yielding the same result as in the experiment with CYA (E).

DISCUSSION

In LLC-MDR1, the accumulation of vinblastine was greatly decreased compared to LLC-PK1 as reported by Horio *et al.* [15], indicating the P-glycoprotein was responsible for extruding the chemical. Although it was possible that the values for accumulation were the result of adsorption in the cell membrane but not that of uptake, the decrease in accumulation coincided with the result of the ID_{50}. The ID_{50} of vinblastine in LLC-MDR1 was greater by 52-fold than to that in LLC-PK1 in the present experiment, and its ID_{50} was within the range mentioned in the previous report [26], so that the decreased accumulation reflected the increased ID_{50}.

The increase in the accumulation of vinblastine by adding CYA in LLC-MDR1 might be considered due to the inhibition of the extrusion through P-glycoprotein binding with CYA as reported earlier [19, 25]. Nevertheless, the effect of CYA was detected not only in LLC-MDR1 but also in LLC-PK1, and the absolute magnitude of the inhibition with CYA was greater in LLC-PK1 than in LLC-MDR1. P-glycoprotein was detected only on the apical side in the monolayer of LLC-MDR1, but it was not detected in LLC-PK1 with anti-human P-glycoprotein antibody which is able to detect porcine P-glycoprotein [30]. Therefore, CYA might inhibit

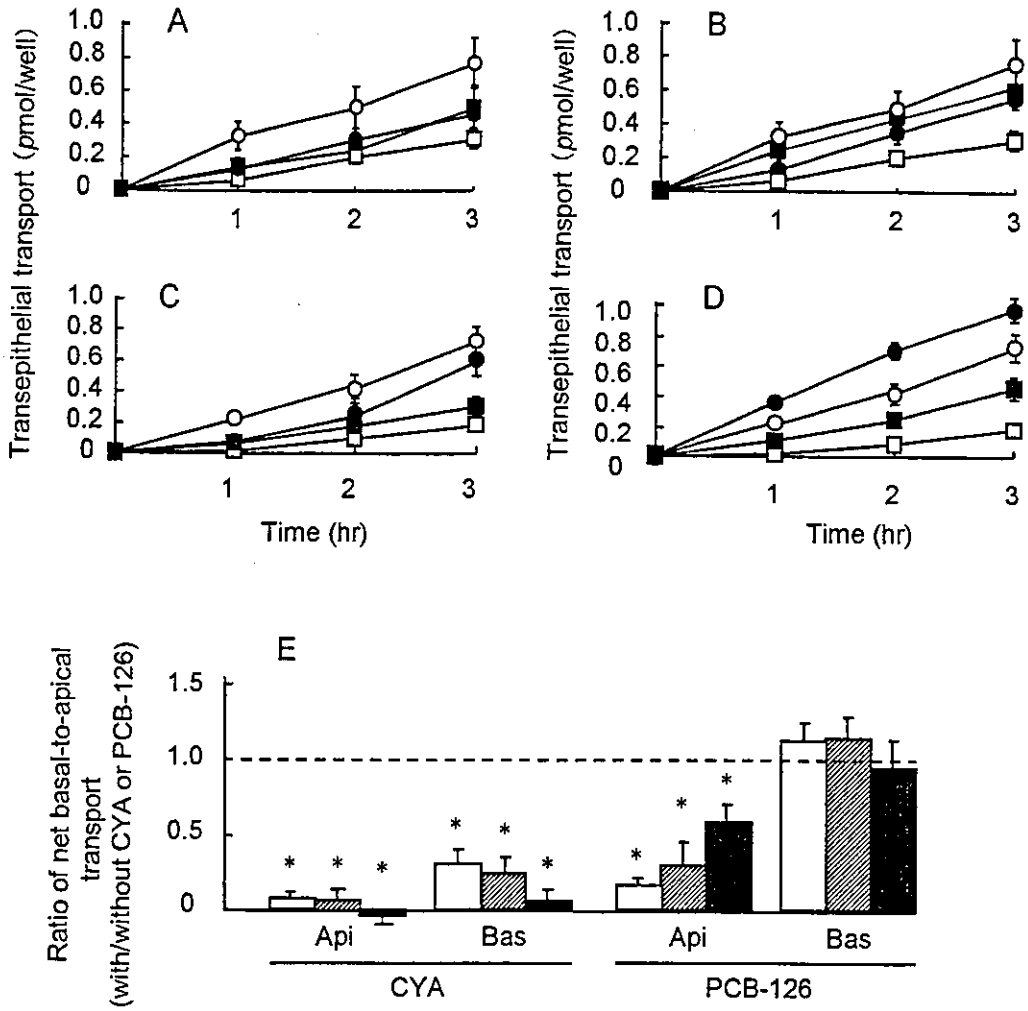

Fig. 3. Effects of CYA (A, B, filled symbols) and PCB-126 (C, D, filled symbols) at the apical (A, C) or basal side (B, D) on the basal-to-apical (circles) and apical-to-basal (squares) transepithelial transport of vinblastine in LLC-PK1, and ratios of the net basal-to-apical transports without and with CYA or PCB-126 on the apical (Api, slanted columns) or basal (Bas, filled columns) side (E). Values are the means and SD of four experiments. *, Statistical significance of transport without and with CYA or PCB-126 on the apical and basal sides in LLC-PK1 ($p<0.05$).

some drug extrusion system other than P-glycoprotein in LLC-PK1. There are many drug extrusion systems in cell membranes [4, 14], so that endogenous drug extrusion systems might be inhibited by binding with CYA in LLC-PK1. Since the effect of CYA on the accumulation in LLC-MDR1 was smaller than that in LLC-PK1, the inhibition of CYA on the drug extrusion though P-glycoprotein was not defined in this experiment.

By adding PCB-126, an inhibition of the decrease in drug accumulation was also detected, suggesting that PCB-126 might act as an inhibitor of that drug extrusion system. Since Co-PCBs seem to readily accumulate in a lipophilic cell membrane, there is the possibility that Co-PCBs act as the binding substrate of P-glycoprotein and hamper drug extrusion in human and animal cell membranes [21]. As a matter of fact, the chemicals of which very little was transported were potent inhibitors of the transport of other transport substrates. One of the congeners of Co-PCBs, 3, 3', 4, 4'-tetrachlorobiphenyl (PCB-77), was hardly transported at all in the monolayer of LLC-MDR1 and was highly accumulated in the cells [9, 10]. Therefore, there is the possibility that Co-PCBs may hamper transport of drugs or metabolites through some drug extrusion systems including P-glycoprotein in cell membranes, thus exerting adverse effects on the cells. However the effect of PCB-126 on the transport in LLC-MDR1 was less than that in LLC-PK1, so that the effect might be due not to the inhibition of P-glycoprotein, but to another drug extrusion system.

The net basal-to-apical transport of vinblastine in the monolayer cells increased in LLC-MDR1 compared to LLC-PK1 as reported earlier [10, 19], thus indicating that the P-glycoprotein was responsible for the net basal-to-api-

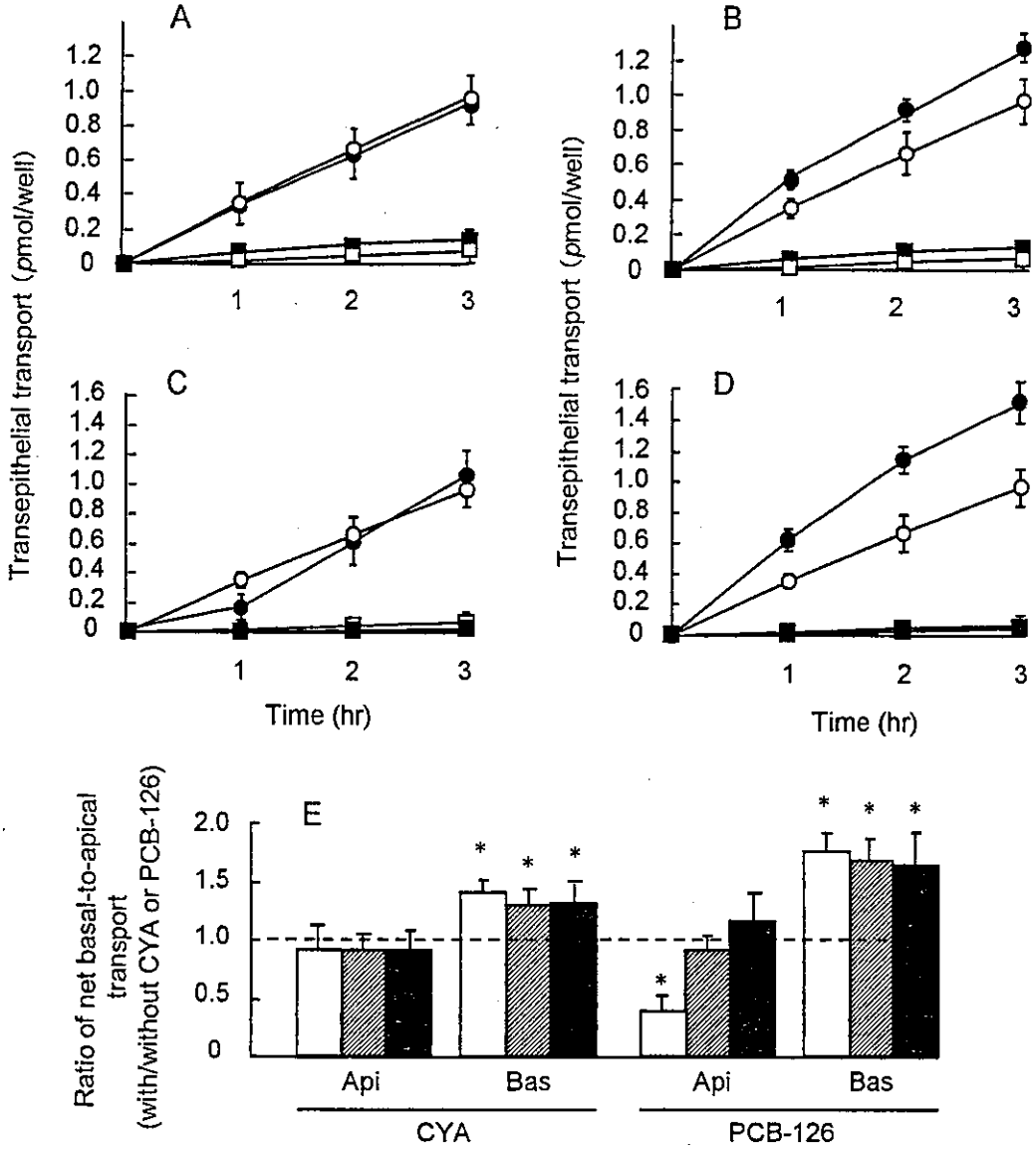

Fig. 4. Effect of CYA (A, B, filled symbols) and PCB-126 (C, D, filled symbols) at the apical (A, C) or basal side (B, D) on the basal-to-apical (circles) and apical-to-basal (squares) transepithelial transport of vinblastine in LLC-MDR1, and ratios of the net basal-to-apical transports without (Cont) and with CYA or PCB-126 on the apical (Api, slanted columns) or basal (Bas, filled columns) side (E). Values are the means and SD of four experiments. *, Statistical significance of the transport without and with CYA or PCB-126 on the apical and basal sides in LLC-MDR1 ($p<0.05$).

cal transepithelial transport. By adding CYA and PCB-126 to the basal side of the monolayer of LLC-MDR1, the basal-to-apical transports were increased, thereby increasing the net basal-to-apical transepithelial transports. This might be due to the enhanced uptake of the chemical on the basal side by the inhibition of some drug extrusion systems. There might be an endogenous drug transport system on the basal side which was inhibited by CYA or PCB-126 in LLC-MDR1. In LLC-PK1, the basal-to-apical transport was also enhanced with PCB-126 on the basal side, but not with CYA, suggesting that the effects of PCB-126 and CYA on a drug transport system may differ from each other.

A decrease in the basal-to-apical transport and an increase in the apical-to-basal transports by adding CYA and PCB126 on the apical side in LLC-PK1 might be interpreted as an inhibition of the extrusion of vinblastine by these chemicals on the apical side. Therefore, the net basal-to-apical transport was reduced by these inhibitors on the apical side in LLC-PK1, though its magnitude was decreased after a 3-hr incubation with PCB126 (Fig. 3-C). Therefore, PCB-126 might affect the apical side at an early stage, then the chemical moves to the basal side and ham-

pers the extrusion of the chemical on that side at a later stage in LLC-MDR1, so that this influx on the basal side might increase later, increasing the net basal-to-apical transport. In LLC-MDR1, a similar effect was also detected when PCB-126 was added on the apical side (Fig. 4-C), though no effect was found subsequently. The apical-to-basal transport was basically very low in LLC-MDR1 (Fig. 4), and the absolute magnitude of the inhibition of the drug extrusion was not so high (Fig. 2A), so that the minor effect on the apical side might fail to affect the net basal-to-apical transport due to the considerable magnitude of the basal-to-apical transport.

In this experiment, the effects of CYA and PCB-126 on the accumulation or transepithelial transport in LLC-MDR1 and LLC-PK1 were different; in most cases, the effects were less in the former cells than in the latter. One interpretation would be that these inhibitions affected mainly transport systems other than P-glycoprotein, and all drug extrusion systems were modified in LLC-MDR1 due to the transfection of the vector with the inserted human P-glycoprotein gene. Sometimes these unexpected effects were observed after gene transfection. For example, in the cells transfected with the K-Cl cotransporter (KCC) gene, ion transport activity other than KCC was enhanced [13]. This was considered to be due to the modification of total gene expression by the transfection of one gene, or the change in the relationship or crosstalk among the protein induced and the other endogenous proteins.

In conclusion, PCB-126 inhibited the accumulation and transepithelial transport of vinblastine as effectively as CYA did, possibly due to an inhibition of drug extrusion systems by PCB-126. Both PCB-126 and the other Co-PCBs are highly lipophilic and accumulated in animal and human organs [1, 3, 12, 22]. Therefore, Co-PCBs may inhibit the function of the drug extrusion systems in many organs, as shown in this experiment. Though the principal toxicities of Co-PCBs are induced by binding with AhR [18, 24], the effects of Co-PCBs on drug transport systems have to be considered one of the possible causes of the adverse effects of these chemicals.

ACKNOWLEDGEMENTS . This work was partly supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (11839027) and from The Project of the High-Tech Research Center, Azabu University.

REFERENCES

1. Asplund, L., Sevensson, B-G., Nilsson, A., Eriksson, U., Jansson, B., Jensen, S., Wideqvist, U. and Skerfving, S. 1994. Polychlorinated biphenyls, 1,1,1-trichloro-2,2-bis)p-chlorophenyl)ethane(p,p'-DDT) and 1,1-dichloro-2,2- bis(p-chlorophenyl)-ethylene(p,p'-DDE) in human plasma related to fish consumption. *Arch. Environ. Health* **49**: 477–486.
2. Annoura, T., Sasawatari, S., Matsutani, H., Ikeda, T., Ueda, K. and Fujise, H. 2001. Change in the drug execratively of human mutant P-glycoprotein expressing cells, KB3. *Vet. Biochem.* **38**: 27–34 (Abstract in English).
3. Bates, M.N., Hannah, D.J., Buckland, S.J., Taucher, J.A. and van Maanen, T. 1994. Chlorinated organic contaminants in breast milk of New Zealand women. *Environ. Health Perspect.* **102** (*Suppl.* 1): 211–217.
4. Borst, P., Evers, R., Kool, M. and Wijnholds, J. 1999. The multidrug resistance protein family. *Biochim. Biophys. Acta* **1461**: 347–357.
5. Barnes, K.M., Dickstein, B., Cutler, G.B. Jr., Fojo, T. and Bates, S.E. 1996. Steroid transport, accumulation, and antagonism of P-glycoprotein in multidrug-resistant cells. *Biochemistry* **35**: 4820–4827.
6. Burton, J.E., Dorociak, I.R., Schwedler, T.E. and Rice, C.D. 2002. Circulating lysozyme and hepatic CYP1A activities during a chronic dietary exposure to tributyltin (TBT) and 3,3',4,4',5-pentachlorobiphenyl (PCB-126) mixtures in channel catfish, *Ictalurus punctatus*. *J. Toxicol. Environ. Health A* **65**: 589–602.
7. Cogliano, V.J. 1998. Assessing the cancer risk from environmental PCBs. *Environ. Health Perspect.* **106**: 317–323.
8. Connor, K., Ramamoorthy, K., Moore, M., Mustain, M., Chen, I., Safe S., Zacharewski, T., Gillesby, B., Joyeux, A. and Balaguer, P. 1997. Hydroxylated polychlorinated biphenyls (PCBs) as estrogens and antiestrogens: structure-activity relationships. *Toxicol. Appl. Pharmacol.* **145**: 111–123.
9. Fujise, H., Annoura, T., Sasawatari, S., Ikeda, T. and Ueda, K. 2000. Transepithelial transport and cellular accumulation of Co-PCBs in porcine kidney cell line, LLC-PK1, and its transformant cells transfected with human multi-drug resistant (MDR) gene. *Organohalogen Compounds* **49**: 269–272.
10. Fujise, H., Annoura, T., Sasawatari, S., Ikeda, T. and Ueda, K. 2002. Transepithelial transport and cellular accumulation of steroid hormones and polychlorobiphenyl in porcine kidney cells expressed with human P-glycoprotein. *Chemosphere* **46**: 1505–1511.
11. Fujise, H., Sasawatari, S., Annoura, T., Ikeda, T. and Ueda, K. 2004. 3,3',4,4',5-pentachlorobiphenyl inhibits drug efflux through P-glycoprotein in KB-3 cells expressing mutant human P-glycoprotein. *J. Biomed. Biotechnol.* (In press).
12. Furst, P., Furst, C. and Wilmers, K. 1994. Human milk as a bioindicator for body burden of PCDDs, PCDFs, organochlorine pesticides, and PCBs. *Environ. Health Perspect.* **102**: 187–193.
13. Gillen, C.M. and Forbush, III, B. 1999. Functional interaction of the K-Cl cotransporter (KCC1) with the Na-K-Cl cotransporter in HEK-293 cells. *Am. J. Physiol.* **276**: C328–336.
14. Gottesman, M.M. and Pastan, I. 1993. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* **62**: 385–427.
15. Horio, M., Pastan, I., Gottesman, M.M. and Handler, J.S. 1990. Transepithelial transport of vinblastine by kidney-derived cell lines. Application of a new kinetic model to estimate in situ Km of the pump. *Biochim. Biophys. Acta* **1027**: 116–122.
16. Jansen, H.T., Cooke, P.S., Porcelli, J., Liu, T.C. and Hansen, L.G. 1993. Estrogenic and antiestrogenic actions of PCBs in the female rat: *in vitro* and *in vivo* studies. *Reprod. Toxicol.* **7**: 237–248.
17. Jacobson, J.L. and Jacobson, S.W. 1997 Evidence for PCBs as neurodevelopmental toxicants in humans. *Neurotoxicology* **18**: 415–424.
18. Kafafi, S.A., Afeefy, H.Y., Ali, A.H., Said, H.K., Abd-Elazem, I.S. and Kafafi, A.G. 1993. Affinities for the aryl hydrocarbon receptor, potencies as aryl hydrocarbon hydroxylase inducers

and relative toxicities of polychlorinated biphenyls - a congener specific approach. *Carcinogenesis* **14**: 2063–2071.

19. Kusunoki, N., Takara, K., Tanigawara, Y., Yamauchi, A., Ueda, K., Komada, F., Ku, Y., Kuroda, Y., Saitoh, Y. and Okumura, K. 1998. Inhibitory effects of a cyclosporine derivative, SDZ PSC 833, on transport of doxorubicin and vinblastine via human P-glycoprotein. *Jpn. J. Cancer Res.* **89**: 1220–1228.
20. Pantaleoni, G.C., Fanini, D., Sponta, A.M., Palumbo, G., Giorgi, R. and Adams, P.M. 1988. Effects of maternal exposure to polychlorobiphenyls (PCBs) on F1 generation behavior in the rat. *Fundam. Appl. Toxicol.* **11**: 440–449.
21. Saeki, T., Ueda, K., Tanigawara, Y., Hori, R. and Komano, T. 1993. Human P-glycoprotein transports cyclosporine A and FK506. *J. Biol. Chem.* **268**: 6077–6080.
22. Safe, S.H. 1994. Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses and implications for risk assessment. *Crit. Rev. Toxicol.* **24**: 87–149.
23. Safe, S.H. 1990. Polychlorinated biphenyls, dibenzo-p-dioxins, dibenzofurans and related compounds: environmental and mechanistic considerations which support the development of Toxic Equivalency Factors. *CRC Crit. Rev. Toxicol.* **21**: 51–88.
24. Shimada, T., Inoue, K., Suzuki, Y., Kawai, T., Azuma, E., Nakajima, T., Shindo, M., Kurose, K., Sugie, A., Yamagishi, Y., Fujii-Kuriyama, Y. and Hashimoto, M. 2002. Aryl hydrocarbon receptor-dependent induction of liver and lung cytochromes P450 1A1, 1A2 and 1B1 by polycyclic aromatic hydrocarbons and polychlorinated biphenyls in genetically engineered C57BL/6J mice. *Carcinogenesis* **23**: 1199–1207.
25. Tamai, I. and Safa, A.R. 1990. Competitive interaction of cyclosporines with the Vinca alkaloid-binding site of P-glycoprotein in multidrug-resistant cells. *J. Biol. Chem.* **265**: 16509–16513.
26. Tanaka, K., Hirai, M., Tanigawara, Y., Ueda, K., Takano, M., Hori, R. and Inui, K. 1997. Relationship between expression level of P-glycoprotein and daunorubicin transport in LLC-PK1 cells transfected with human MDR1 gene. *Biochem. Pharmacol.* **53**: 741–746.
27. Tanigawara, Y., Okamura, N., Hirai, M., Yasuhara, M., Ueda, K., Kioka, N., Komano, T. and Hori, R. 1992. Transport of digoxin by human P-glycoprotein expressed in a porcine kidney epithelial cell line (LLC-PK1). *J. Pharmacol. Exp. Ther.* **263**: 840–845.
28. Thiebaut, G., Tsuruo, T., Hamada, H., Gottesman, M.M. Pastan, I. and Willingham, M.C. 1987. Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues. *Proc. Natl. Acad. Sci. U.S.A.* **84**: 7735–7738.
29. Ueda, K., Cardarelli, C., Gottesman, M.M. and Pastan, I. 1987. Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc. Natl. Acad. Sci. U.S.A.* **84**: 3004–3008.
30. Ueda, K., Okamura, N., Hirai, M., Tanigawara, Y., Saeki, T., Kioka, N., Komano, T. and Hori, R. 1992. Human P-glycoprotein transports cortisol, aldosterone, and dexamethasone, but not progesterone. *J. Biol. Chem.* **267**: 24248–24252.
31. Wormke, M., Castro-Rivera, E., Chen, I. and Safe, S. 2000. Estrogen and aryl hydrocarbon receptor expression and crosstalk in human Ishikawa endometrial cancer cells. *J. Steroid Biochem. Mol. Biol.* **72**: 197–207.

uraemic pruritus. Our case confirms the efficacy of this approach in a haemodialysis patient with very severe clinical manifestations of the disease, which led her to attempt suicide. In this unblinded single patient we cannot exclude a placebo effect, which can be particularly relevant in patients with pruritus. However, no placebo effect could be observed with all the other treatments (antihistamines, steroids and UVB light) carried out in this patient. In addition, a consistent pharmacological effect from cyclosporin is indicated by the response to challenge, withdrawal and rechallenge.

Although the potential side effects of an immunosuppressive drug in dialysis patients should be kept in mind, our results indicate that cyclosporin treatment might be a new effective approach to severe uraemic pruritus refractory to conventional treatment modalities, provided that appropriate patients are selected and careful monitoring is performed. Our observation and hypothesis need to be confirmed by a placebo-controlled double-blind trial.

Conflict of interest statement. None declared.

Nephrology and Dialysis Unit
[1]Hospital of Chioggia, Venice
[2]San Paolo Hospital, Milan
Italy
Email: gallieni@iol.it

Maria Fusaro[1]
Giorgio Munaretto[1]
Michela Spinello[1]
Maurizio Gallieni[2]

1. Berth-Jones J, Graham-Brown RA, Marks R *et al.* Long-term efficacy and safety of cyclosporin in severe adult atopic dermatitis. *Br J Dermatol* 1997; 136: 76–81
2. Mettang T, Pauli-Magnus C, Alscher DM. Uraemic pruritus: new perspectives and insights from recent trials. *Nephrol Dial Transplant* 2002; 17: 1558–1563
3. Peer G, Kivity S, Agami O *et al.* Randomised crossover trial of naltrexone in uremic pruritus. *Lancet* 1996; 348: 1552–1554
4. Pauli-Magnus C, Mikus G, Alscher DM *et al.* Naltrexone does not relieve uremic pruritus: results of a randomized, placebo-controlled crossover-study. *J Am Soc Nephrol* 2000; 11: 514–519

DOI: 10.1093/ndt/gfh131

Treatment of digoxin intoxication model by hybrid-kidney with hollowfibre module for clinical haemodialysis

Sir,

Although haemoperfusion is frequently used for the treatment of drug intoxication, this has some disadvantages and its use is limited [1]. We have previously reported a unique hybrid-type artificial kidney by culturing the immortalized renal proximal tubule cells with the introduction of multidrug resistance protein (MDR)-1 in the hollowfibre module for cell culture [2]. Moreover, we scaled up the system by connecting 10 modules in parallel and successfully treated dogs with digoxin intoxication, a substrate of MDR-1 [3]. Although this device was effective for the dog, we should further increase the number of modules connected for future clinical use. Here, we succeeded in scaling up the 'hybrid-kidney' by utilizing a single clinically used haemodialyzer and evaluated the efficacy for drug removal *in vitro* and in dogs with digoxin intoxication.

We used the same cell line, into which cDNA of human MDR-1 [4] was introduced. This clone, named PCTL-MDR, possesses about 100 times larger K_m and V_{max} values for digoxin than control cells, named PCTL [2]. A hollowfibre module available for clinical haemodialysis (APS-08S; Asahi Medical, Tokyo, Japan) made of polysulfone with a surface area of 0.8 m^2 was purchased. We inoculated the cells onto the hollowfibre by an almost identical method to that reported previously [2,3]. Thus, 5.4×10^9 cells were injected on the pericapillary side of the module and cultured for 1 week in a CO_2 incubator at 37°C. After incubation, transport of digoxin and inulin from the capillary to pericapillary side were evaluated *in vitro*. We found that >85% of perfused digoxin was transported from the capillary to pericapillary side by the system with PCTL-MDR, while such transport was only ~10% with PCTL and 20% without cells, respectively. Inulin concentration was not reduced on the venous side by the system with the cells, indicating that leakage did not occur. Next, we applied this to the dog model with digoxin intoxication [3,5]. Using PCTL-MDR, the digoxin concentration decreased to the therapeutic level at the end of a 3-h treatment. Although treatment with PCTL reduced digoxin concentration, the observed decrease was significantly smaller than with PCTL-MDR (Figure 1). Estimated digoxin clearance with PCTL-MDR was 31 ± 2 ml/min. Slight leukocytopaenia and thrombocytopaenia, and elevated activity of circulating granulocyte elastase, was detected. However, the magnitude of these parameters was similar between three trials, and dogs tolerated this treatment well. Comparing digoxin clearance in the present experiments with that of adult [6], we propose to treat patients by increasing surface area of the single haemodialyzer to 2 m^2, which is now commercially available. Thus, the present results suggest that our scaled-up module has sufficient capacity to treat digoxin-intoxicated patients, especially when complicated by renal failure. It might be useful to apply it to various types of artificial hybrid-kidneys with different types of cells for the treatment of patients in the future.

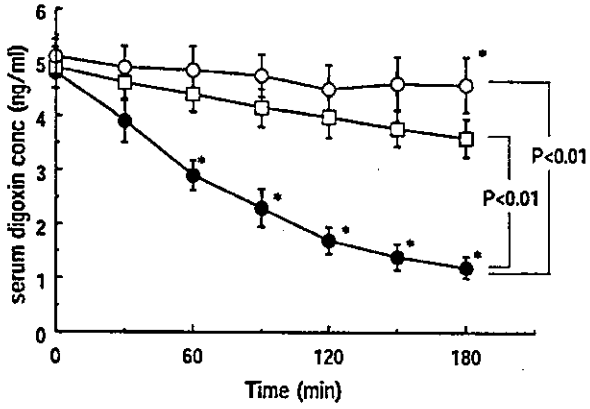

Fig. 1. Change of serum digoxin concentration at just before (arterial) the hollowfibre module during treatment. Mean ± SE, $n = 6$; closed circles, PCTL-MDR; open circles, PCTL; open squares, without cell; $^*P < 0.05$ *vs.* without cell.

Conflict of interest statement. None declared.

Department of Pharmacology
Division of Clinical Pharmacology
Jichi Medical School
Tochigi
Japan
Email: tsuru@jichi.ac.jp

Shuichi Tsuruoka
Kenta Nishiki
Michi Wakaumi
Ning Wang
Hisashi Yamamoto
Hitoshi Ando
Masashi Imai
Akio Fujimura

1. Gurland H, Samtleben W, Lysaght M *et al.* Hemoperfusion. In: Gurland H, ed. *Replacement of Renal Function by Dialysis.* Kluwer Academic, Dordrecht, The Netherlands, 1996; 485–500
2. Tsuruoka S, Sugimoto KI, Ueda K *et al.* Removal of digoxin and doxorubicin by multidrug resistance protein-overexpressed cell culture in hollow fiber. *Kidney Int* 1999; 56: 154–163
3. Tsuruoka S, Nishiki K, Sugimoto K *et al.* Specific therapy of digoxin intoxication in dogs by hybrid kidney overexpressing multidrug resistance protein. *Kidney Int* 2002; 62: 1332–1337
4. Ueda K, Cardarelli C, Gottesman MM *et al.* Expression of a full-length cDNA for the human 'MDR1' gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc Natl Acad Sci USA* 1987; 84: 3004–3008
5. Tsuruoka S, Osono E, Nishiki K *et al.* Removal of digoxin by column for specific adsorption of beta(2)-microglobulin: a potential use for digoxin intoxication. *Clin Pharmacol Ther* 2001; 69: 422–430
6. Aronson J. Clinical pharmacokinetics of digoxin 1980. *Clin Pharmacokinet* 1980; 5: 137–149

DOI: 10.1093/ndt/gfh135

Peritoneal dialysis-related peritonitis with bacteraemia due to *Erysipelothrix rhusiopathiae*

Sir,

Case. A 49-year-old bricklayer with end-stage renal failure secondary to adult polycystic kidney disease had been on continuous ambulatory peritoneal dialysis (CAPD) for 1 year. He presented with a 1-day history of abdominal pain, rigors and cloudy bags. Multiple excoriations were present on his hands. The CAPD fluid had a white cell count (WCC) of $>100 \times 10^6$/l with no red cells. The fluid was inoculated into an Oxoid bottle (Oxoid signal blood culture system, Oxoid Ltd, Basingstoke, UK). Filtration was not performed as only 20 ml was received. The peripheral WCC was 12.7×10^9/l. Intraperitoneal vancomycin and gentamicin were commenced. His abdominal pain failed to settle, and by day 4 signs of severe peritonitis were present. Blood cultures were taken and repeat CAPD fluid had a WCC of $>100 \times 10^6$/l. Laparotomy was performed with removal of the Tenchkoff catheter and peritoneal lavage. Purulent free fluid was present throughout the abdominal cavity. Intravenous ciprofloxacin 200 mg bd was started according to local policy to cover possible *Pseudomonas* peritonitis or other bowel-related organisms.

On day 5, a Gram-positive rod was grown from the first CAPD fluid and identified as *Erysipelothrix rhusiopathiae*, using the API Coryne (BioMerieux UK Ltd, Basingstoke, UK). It was resistant to vancomycin and gentamicin but sensitive to ciprofloxacin, penicillin, erythromycin and cefuroxime. The same organism was identified subsequently from blood cultures, although the second CAPD fluid and an intraoperative swab taken from the CAPD tunnel were both culture negative. Symptoms resolved, a vascath was inserted for haemodialysis, and he was discharged following 12 days of ciprofloxacin. He remained on haemodialysis until his death from unrelated causes 8 months later.

Comment. Erysipelothrix rhusiopathiae is a Gram-positive rod which may be confused with *Lactobacillus*, *Corynebacterium* or *Enterococcus* spp on the basis of morphology and biochemical tests [1]. It has a wide geographical distribution and has been reported from a variety of animals as a commensal or pathogen [1]. It causes severe disease in domestic pigs, poultry and sheep. Colonization has been reported in fish, shellfish and birds. The organism can survive in soil for several months [1].

Infection in humans usually follows cutaneous inoculation so is often related to occupational exposure, i.e. butchers, fishmongers and veterinarians [1]. There are three main clinical syndromes. The most common is erysipeloid, a painful localized violaceous skin lesion. Less commonly described are a more severe, diffuse cutaneous form and also a bacteraemic illness usually associated with endocarditis [1]. Most cases of bacteraemia without endocarditis have occurred in immunocompromised hosts [2].

Most strains of *E.rhusiopathiae* are sensitive to penicillins, cephalosporins, imipenem, clindamycin, erythromycin and ciprofloxacin. Penicillin is the antibiotic of choice. Most are resistant to vancomycin, aminoglycosides, teicoplanin and trimethoprim-sulfamethoxazole. The glycopeptide resistance is of particular note as vancomycin is often used as empiric treatment of Gram-positive bacteraemia or endocarditis, and intraperitoneal vancomycin and gentamicin is a common first-line treatment for CAPD peritonitis [3]. Most Gram-positive rods are vancomycin sensitive. Only one reported case of bacteraemia or endocarditis has been treated successfully with ciprofloxacin [4]. Our patient had improved by the time sensitivities were available, so ciprofloxacin was continued instead of switching to penicillin.

This is the second reported case of CAPD peritonitis caused by this organism, and the first European case. The first case occurred in a rancher who cut his hand on a barbed wire fence around an animal enclosure 2 weeks before admission [5]. He became pyrexial with a skin lesion on his hand. Initial treatment was with intravenous gentamicin and intraperitoneal amikacin, changed to intravenous penicillin following isolation of the organism from CAPD fluid. No organisms were seen on the Gram stain. In contrast to our case, blood cultures were sterile and the patient responded to medical treatment.

CAPD peritonitis may occur by several routes. The most common is thought to be intraluminal and is the major route for skin and environmental organisms. The extraluminal (i.e. tunnel migration) route may complicate exit site infections. The transluminal route (i.e. migration across the bowel wall) involves bowel flora. Infection by the haematogenous route may complicate bacteraemia. The intraluminal or haematogenous route seems most likely in this case. No direct animal exposure was documented, but inoculation from a contaminated environmental source may have occurred through excoriated hands.

This case demonstrates the importance of identifying and determining the sensitivity of all isolates from CAPD fluids. Ciprofloxacin may be used to treat *E.rhusiopathiae* bacteraemia in patients with a severe penicillin allergy.

Conflict of interest statement. None declared.

[1]Department of Microbiology Susan C. Hardman[1]
[2]Department of Nephrology Susan J. Carr[2]
University Hospitals of Leicester R. Andrew Swann[1]
Leicester, UK
Email: susanhardman@hotmail.com

1. Mandell GL, Bennett JE, Dolin R, eds. *Erysipelothrix rhusiopathiae.* In: *Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases,* 5th edn. Churchill Livingstone, Edinburgh; 2000: 2226–2227
2. Ognibene FP, Cunnion RE, Gill V *et al. Erysipelothrix rhusiopathiae* bacteraemia presenting as septic shock. *Am J Med* 1985; 78: 861–864

Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 488 (2004) 239–245
ELSEVIER
ejp
www.elsevier.com/locate/ejphar

Chronopharmacology of oxacalcitriol in rat model of osteoporosis

Shuichi Tsuruoka*, Michi Wakaumi, Hisashi Yamamoto, Akio Fujimura

Department of Pharmacology, Division of Clinical Pharmacology, Jichi Medical School, 3311 Yakushiji, Minamikawachi, Kawachi, Tochigi 329-0498, Japan

Received 30 October 2003; received in revised form 5 February 2004; accepted 6 February 2004

Abstract

We have previously reported the merits of chronopharmacological effect of 1-α(OH) vitamin D3 in aged stroke-prone spontaneously hypertensive rat (SHRSP), a model of osteoporosis [Eur. J. Pharmacol. 428 (2001) 283.]. In this study, the chronopharmacological effect of 22-oxacalcitriol, a newly developed active vitamin D3 analogue with less calcemic activity, was evaluated by a single and repeated dosing of the drug in aged SHRSP. Animals (7 months old) were kept in rooms with a 12-h light/dark cycle. Single (12.5 μg/kg, i.v.) and repeated (5 μg/kg, i.v. three times a week for 12 weeks) dosing of 22-oxacalcitriol or vehicle was given at either 2 h after lights on (2HALO) or 14 h after lights on (14HALO). The severity of adverse reactions such as the changes of body weight, hypercalcemia and hyperphosphatemia, was significantly mild when the drug was given at 14HALO. Especially, the increase of serum Ca concentration was not detected at 14HALO trial. Serum concentrations of total (protein-bound and unbound) 22-oxacalcitriol and albumin (a major binding protein of the drug) of the 2HALO and 14HALO trials did not significantly differ. The decrease of parathyroid hormone (PTH) concentration was greater in the 14HALO trial while the increase in urinary ratio of Ca to creatinine was greater in the 2HALO trial. The increase in bone density of both femurs at the end of the study was greater in the 14HALO trial. The suppression of urinary excretion of deoxypyridinoline, an index of bone resorption capacity of osteoclast, was greater in the 14HALO trial, which indicates that the efficacy of 22-oxacalcitriol for suppressing bone resorption might vary with the dosing time. This is the first study to show the dosing-time-dependent changes in the efficacy and toxicity of 22-oxacalcitriol in the animal model of osteoporosis. Chronopharmacological differences seem to be more prominent than those of other vitamin D analogues. To use 22-oxacalcitriol at an adequate timing might provide better efficacy and safety than other vitamin D3 analogues for the treatment of osteoporosis.

Keywords: 22-Oxacalcitriol; Osteoporosis; Chronotherapy; Hypercalcemia

1. Introduction

Although active vitamin D analogues are widely used for the treatment of osteoporosis and secondary hyperparathyroidism, the drug-related hypercalcemia and hyperphosphatemia sometimes limit its efficacy (Parfitt, 1988). It is well known that serum Ca and P concentrations show diurnal changes in both humans (Tsuruoka et al., 1999) and rats (Shinoda and Seto, 1985; Shinoda and Stern, 1992). We have previously reported that hypercalcemia and hyperphosphatemia by an active vitamin D3 can be diminished by administration of the drug at night in aged stroke-prone spontaneously hypertensive rats (SHRSP), a model of osteoporosis (Tsuruoka et al., 2001), and patients with secondary hyperparathyroidism (Tsuruoka et al., 1999, 2003). We have also reported that the efficacy of vitamin D3 therapy can be enhanced by the drug administration at night in aged SHRSP (Tsuruoka et al., 2001), 5/6-nephrectomized rats (Tsuruoka et al., 2002), and patients with secondary hyperparathyroidism (Tsuruoka et al., 2003).

Compared to 1,25(OH)2 vitamin D3, the 22-oxacalcitriol (or maxacalcitriol), a new analogue of vitamin D3, is reported to have a less hypercalcemic effect with similar efficacy (Brown et al., 1993; Kubrusly et al., 1993; Farach-Carson et al., 1993). This compound is now used for the treatment of secondary hyperparathyroidism and osteoporosis in clinical situation. However, it remains to be determined whether 22-oxacalcitriol also possesses chronopharmacological effect. It was a purpose of this study to evaluate a dosing-time-dependent change in the effects of 22-oxacalcitriol in aged SHRSP, a rat model of osteoporosis. We obtained some chronopharmacological profiles in its efficacy and adverse reactions, which were compared with our previous results using other vitamin D analogues.

* Corresponding author. Tel.: +81-285-58-7388; fax: +81-285-44-7562.
E-mail address: tsuru@jichi.ac.jp (S. Tsuruoka).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.02.007