

## Polarized cholesterol and phospholipid efflux in cultured gall-bladder epithelial cells: evidence for an ABCA1-mediated pathway

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Gall-bladder epithelial cells (GBEC) are exposed to high concentrations of cholesterol in bile. Whereas cholesterol absorption by GBEC is established, the fate of this absorbed cholesterol is not known. The aim of this study was to determine whether ABCA1 (ATP-binding cassette transporter A1) mediates cholesterol efflux in GBEC. Polarized canine GBEC were cultured on porous membrane filters allowing separate access to apical (AP) and basolateral (BL) compartments. After AP loading of cells with model bile and [<sup>14</sup>C]cholesterol, cholesterol efflux was measured. Cholesterol loading together with 8-bromo-cAMP treatment, which increased ABCA1 expression, led to a significant increase in cholesterol efflux with apolipoprotein A-I (apoA-I) as the acceptor. Cholesterol efflux was observed predominantly into the BL compartment. Similar results were found for phospholipid efflux. Confocal immunofluorescence microscopy showed a pre-

dominantly BL ABCA1 localization. Interestingly, apoA-I added to either the AP or the BL compartments elicited BL lipid efflux with cAMP treatment. No paracellular or transcellular passage of [<sup>125</sup>I]-apoA-I occurred. Ligands for the nuclear hormone receptors liver X receptor  $\alpha$  (LXR $\alpha$ ) and retinoid X receptor (RXR) elicited AP and BL cholesterol efflux, suggesting the involvement of both ABCA1- and non-ABCA1-mediated pathways. In summary, BL cholesterol/phospholipid efflux consistent with an ABCA1-mediated mechanism occurs in GBEC. This efflux pathway is stimulated by cAMP and by LXR $\alpha$ /RXR ligands, and in the case of the cAMP pathway appears to involve a role for biliary apoA-I.

**Key words:** apolipoprotein A-I, bile, nuclear hormone receptor, oxysterol, retinoic acid.

### INTRODUCTION

Bile is the major route of cholesterol excretion from the body [1]. Bile is concentrated during the interdigestive phase in the gall-bladder (GB) and is often supersaturated in terms of cholesterol concentration [2]. Such high levels of cholesterol in GB bile have clinical implications with respect to cholesterol gallstone formation and cholesterosis of the GB wall. Gall-bladder epithelial cells (GBEC) are exposed to high cholesterol concentrations on their apical (AP) surface. Therefore, GBEC are uniquely positioned to play a role in modulating biliary cholesterol concentrations.

Little is known at the cellular and molecular levels about how GBEC interact with biliary cholesterol. The GB epithelium absorbs cholesterol and phospholipids via both passive and active mechanisms [3]. Studies *in vitro* have shown that lipid absorption by the GB epithelium alters cholesterol solubility in bile [4]. The fate of the cholesterol and phospholipid absorbed by GBEC, however, is not known. One possibility is the re-secretion of absorbed cholesterol and phospholipid back into bile, as has been demonstrated in cultured GBEC [5]. Another possibility is that GBEC possess mechanisms to efflux absorbed cholesterol via the basolateral (BL) plasma membrane. Such a pathway would be analogous to the cholehepatic shunt of bile acids mediated by the sodium-dependent bile acid transporter [6]. Indirect evidence for such a transport pathway for biliary cholesterol exists. Plasma lipoproteins take up cholesterol ab-

sorbed by the GB epithelium [7]. Biliary apolipoproteins may also be involved in such a pathway. Apolipoprotein A-I (apoA-I) is present in bile and possesses anti-nucleating properties [8,9]. Biliary apoA-I enhances transfer of cholesterol from the mucosal to the serosal side of cultured GBEC [10]. These results suggest a role for biliary apoA-I in mediating mucosal-to-serosal transport of cholesterol through GB epithelium.

The cholesterol/phospholipid efflux protein ABCA1 [ATP-binding cassette (ABC) transporter A1] may mediate cholesterol transport in GBEC. ABCA1 is a membrane-bound ABC transporter that is mutated in patients with Tangier disease [11–14]. These patients have a defect in reverse cholesterol transport, whereby cholesterol cannot be mobilized from peripheral sites [15]. Patients have very low levels of high-density lipoprotein (HDL) cholesterol, manifest excessive peripheral deposits of cholesterol ester and die prematurely from atherosclerosis [16]. The mutated ABCA1 in patients with Tangier disease cannot efflux cholesterol from a variety of cells on to apoA-I [11,17]. Without this crucial step, mature HDL particles cannot form, and hence cholesterol from peripheral sites cannot be transported to the liver for disposal or re-use.

We therefore set out to determine whether ABCA1 was expressed in polarized GBEC. As prior studies on ABCA1 expression and function had been performed in macrophages and fibroblasts [11,17–19], cells that do not exhibit strict polarization, an additional aim of our studies was to determine the polarity of cholesterol efflux. We also modified the cholesterol-

Abbreviations used: AP, apical; apoA-I, apolipoprotein A-I; apoE, apolipoprotein E; ABC, ATP-binding cassette; BHK, baby hamster kidney; BL, basolateral; 8-Br-cAMP, 8-bromo-cAMP; FBS, fetal bovine serum; GB, gall-bladder; GBEC, gall-bladder epithelial cells; HDL, high-density lipoprotein; SFM, serum-free medium; LXR $\alpha$ , liver X receptor  $\alpha$ ; RXR, retinoid X receptor.

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efflux assay used extensively on macrophages and fibroblasts to make it better suited for GBEC. Radiolabelled cholesterol in model bile was added to the AP compartment of confluent GBEC to mimic cholesterol loading in the GB *in vivo*. The results provide evidence for the presence of functional ABCA1 in GBEC. The predominantly BL localization of ABCA1 suggests that GB epithelium is capable of initiating a cholehepatic pathway that returns cholesterol to the liver.

The nuclear hormone receptors liver X receptor  $\alpha$  (LXR $\alpha$ ) and retinoid X receptor (RXR) have been implicated in cholesterol homeostasis in the liver, intestines and macrophages [20–22]. LXR $\alpha$ /RXR effects are mediated in part by transcriptional control of ABCA1 expression [23,24]. An additional aim of our studies was to determine whether LXR $\alpha$  and RXR are involved in regulating cholesterol efflux in polarized cultured GBEC. As GB epithelium is exposed not only to high levels of cholesterol, but also to a variety of oxysterols in bile on their AP surface [25], we hypothesized that the regulation of ABCA1 and other cholesterol-homoeostatic mechanisms in GBEC are subject to control by oxysterol composition and concentration. As the LXR $\alpha$ /RXR heterodimer is a more potent transcriptional activator of ABCA1 than either receptor alone in other cell types [23,24], we surmised that if GBEC were subject to regulation by these nuclear hormone receptors, then activation by the combination of LXR $\alpha$ /RXR would be a logical focus of investigation.

## EXPERIMENTAL

### Materials

Eagle's minimum essential medium, fetal bovine serum (FBS), trypsin/EDTA, penicillin/streptomycin, L- $\alpha$ -phosphatidylcholine, 8-bromo-cAMP (8-Br-cAMP), 9-*cis*-retinoic acid, 22-(*R*)-hydroxycholesterol and peroxidase-conjugated anti-rabbit IgG were from Sigma (St. Louis, MO, U.S.A.). Vitrogen was from Celtrix (Palo Alto, CA, U.S.A.). Transwell cell-culture plates (diameter, 12 or 24 mm; pore size, 3.0  $\mu$ m) were from Costar (Cambridge, MA, U.S.A.). Taurocholic acid, fatty acid-free BSA and cholesterol were from Calbiochem (La Jolla, CA, U.S.A.). [ $^{14}$ C]cholesterol and [ $^3$ H]mannitol were from NEN (Boston, MA, U.S.A.). [*methyl*- $^3$ H]choline chloride and enhanced chemiluminescence detection reagents (ECL-Plus) were from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). Alexa 568 goat anti-rabbit IgG was from Molecular Probes (Eugene, OR, U.S.A.). The chicken polyclonal anti-canine Na $^+$ /K $^+$ -ATPase antibody was from Abcam (Cambridge, U.K.).

### Cell culture

Epithelial cells were isolated from canine GB, as described previously [26]. Stock cultures were grown on 100 mm plates with 2 ml of Vitrogen gel (1:1 mixture of Vitrogen and media) in Eagle's minimum essential medium supplemented with 10% FBS, 2 mM L-glutamine, 20 mM Hepes, 100 i.u./ml penicillin and 100  $\mu$ g/ml streptomycin. Media was changed twice a week and the cells were maintained in a 37 °C incubator with 5% CO $_2$ . Cells were passaged when confluent (every 7–10 days) using trypsin (2.5 g/l) and EDTA (1 g/l). Cultures on Transwell inserts were performed under the same conditions as stock cultures except that no Vitrogen was used. Baby hamster kidney (BHK) cells expressing ABCA1 under the control of a mifepristone-inducible gene were prepared as described previously [27]. These cells express only trace amounts of ABCA1 unless treated with mifepristone.

### Preparation of model bile and apoA-I

Model bile with a cholesterol saturation index of 1.5 was prepared as described in [28]. Taurocholic acid was dissolved in methanol/water (85:15, v/v), and mixed with lecithin (L- $\alpha$ -phosphatidylcholine) and purified recrystallized cholesterol in chloroform. The solvent was then evaporated under nitrogen, and the residue was lyophilized. Model bile was stored at –70 °C until use. For experiments, model bile was dissolved in 5 ml of PBS, pH 7.4, and equilibrated overnight at 56 °C. Model bile was then diluted 1:10 in serum-free medium (SFM) and sterilized through a 0.45  $\mu$ m filter. Human de-lipidated apoA-I was purified from HDL as described in [17].

### Cholesterol and phospholipid efflux assays

GBEC were cultured on 12 mm-diameter Transwell inserts without Vitrogen until confluent. An equilibrated mixture of model bile and [ $^{14}$ C]cholesterol (0.12  $\mu$ Ci/ml) or [ $^3$ H]choline (2  $\mu$ Ci/ml) was added to the AP compartment for 48 h. 8-Br-cAMP (1 mM) was added to both compartments to certain Transwells for the latter 24 h. Cells were then washed three times with PBS/0.2% BSA, and once with SFM containing 0.2% BSA. A subsequent incubation for 24 h was performed with SFM containing 0.2% BSA with 1 mM 8-Br-cAMP added to both sides and/or 10  $\mu$ g/ml de-lipidated human apoA-I to either the AP or BL compartments as indicated. At selected times, medium was collected and centrifuged to remove cell debris, and cells were washed twice with cold PBS/0.2% BSA and twice with PBS. Scintillation fluid was added to the collected medium and scintillation counting performed. Cell layers were dissolved with tissue solubilizer (TS-2; Research Products, Mt. Prospect, IL, U.S.A.) for 24 h, and counted following addition of scintillation fluid. Results are shown as percentage efflux [(counts in AP or BL media/total counts in cells and in media from both compartments)  $\times$  100].

For cholesterol-efflux assays using ligands for LXR $\alpha$ /RXR, 10  $\mu$ M 9-*cis*-retinoic acid and 10  $\mu$ M 22-(*R*)-hydroxycholesterol in ethanol (0.2% v/v) were added to both the AP and BL compartments following model bile treatment of confluent cells as described above. Incubation was carried out for 24 h, then the same concentration of these ligands was continued when apoA-I was added.

### Immunofluorescence studies

GBEC were cultured on 12 mm-diameter Transwell inserts until confluent. Model bile was added to the AP compartment for 48 h with or without 1 mM 8-Br-cAMP. 8-Br-cAMP was added to both the AP and BL compartments for 24 h prior to fixation. Selected cells were treated with 8-Br-cAMP without model bile for 24 h. Cells were washed twice with ice-cold PBS, and the Transwell membranes were cut out and placed on a culture plate. Cells were fixed in methanol/acetone (1:1, v/v), incubated at –20 °C for 15 min, and washed with ice-cold PBS. Blocking buffer (PBS with 1% BSA) was added for 15 min. Primary antibody (C-terminal peptide-specific polyclonal rabbit ABCA1 antibody [11]; a gift from CV Therapeutics, Palo Alto, CA, U.S.A.) or pre-immune serum was applied for 60 min. Following washing with PBS, secondary antibody (Alexa 568 goat anti-rabbit IgG) was added for 60 min. Cells were washed three times with PBS and sealed in mounting media. A Zeiss Axioplan fluorescence microscope fitted with a Hamamatsu C4880 integrating digital camera and MCID software (Imaging

Research, St. Catharines, Ontario, Canada) were used for image acquisition and analysis at 40 $\times$  magnification.

In order to demonstrate specificity of the ABCA1 antibody on immunofluorescence studies, BHK cells expressing ABCA1 using the mifepristone-inducible GeneSwitch System were used [27]. Control BHK cells were derived from the same clonal line but lacked the ABCA1 plasmid. Cells were cultured on glass coverslips in Dulbecco's modified Eagle's medium containing 10% FBS, and treated for 24 h with 10 nM mifepristone. Immunofluorescence was performed as described above, using the ABCA1 antibody and the Alexa 568 goat anti-rabbit IgG secondary antibody.

### Western blotting

GBEC were cultured to confluency on Transwell inserts. Model bile was added to the AP compartment for 48 h with or without 1 mM 8-Br-cAMP added to both sides of certain Transwells for the latter 24 h. After changing to SFM, 1 mM 8-Br-cAMP was re-added to both compartments for 24 h to the indicated cells. The AP and BL media were collected, and concentrated with a Centricon 10 microconcentrator (Amicon, Beverly, MA, U.S.A.). Cells were harvested with SDS loading buffer (250 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 0.006% Bromophenol Blue and 2%  $\beta$ -mercaptoethanol). Protein content of media and cell extracts was measured by the Lowry method. SDS/PAGE was performed with a 4% stacking gel and a 6% resolving gel, followed by transfer to PVDF membrane. The membrane was blocked with 1% BSA in PBS/Tween-20 (0.05%, v/v) at 4 $^{\circ}$ C for 16 h, and then incubated with a rabbit polyclonal ABCA1 antibody for 1 h at room temperature. The membrane was then washed with 0.05% Tween-20 in PBS and incubated with peroxidase-conjugated anti-rabbit or anti-goat IgG for 1 h at room temperature. The membrane was washed and incubated with the ECL-Plus detection system for 5 min, and autoradiography performed. The signal intensities for specific bands on the Western blots were quantified using NIH Image J Density Analysis Software (version 1.20).

### Permeability and labelling assays with $^{125}$ I-apoA-I and [ $^3$ H]mannitol

GBEC were cultured on 12-mm diameter Transwell inserts until confluent. Model bile was added to the AP side for 48 h. 8-Br-cAMP (1 mM) was added to both sides to certain cells for the latter 24 h. Cells were then washed three times with PBS/0.2% BSA and once with SFM/0.2% BSA. Cells were then incubated with SFM/0.2% BSA containing 10  $\mu$ g/ml  $^{125}$ I-apoA-I and unlabelled apoA-I. Following 24 h of incubation, media from the AP and BL compartments was collected, and  $\gamma$ -counting performed. Cells were washed twice with ice-cold PBS/0.2% BSA and twice with PBS. Cell layers were dissolved in 0.1 M NaOH, counted and aliquots taken for protein quantification. This experiment was performed simultaneously with 2  $\mu$ Ci/ml [ $^3$ H]mannitol as a control instead of  $^{125}$ I-apoA-I.

### Confocal laser-scanning immunofluorescence microscopy

Slides were prepared as described for immunofluorescence microscopy studies. Scanned images were acquired with 400 $\times$  optical and 3 $\times$  digital magnification using a laser-scanning spectral confocal microscope system (Leica DM-R upright fluorescence microscope and Leica TCS-SP confocal scanner). For the co-localization studies, the same slides were treated with the ABCA1 antibody and the Na $^+$ /K $^+$ -ATPase antibody, and scanning confocal images obtained. In these studies, the sec-

ondary antibodies used were FITC-conjugated goat anti-rabbit and Rhodamine-conjugated donkey anti-chicken, respectively.

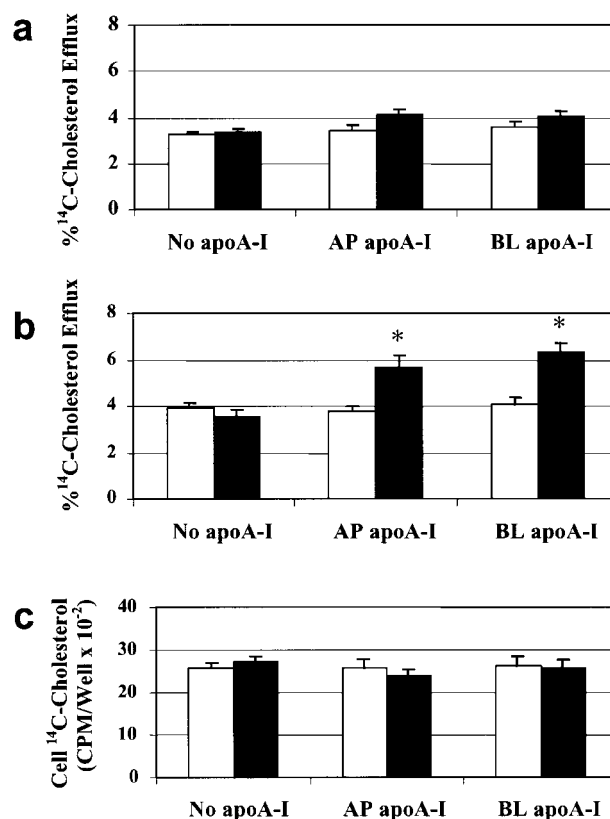
### Statistical analysis

A minimum of three separate incubations (each done in at least duplicate wells) was performed for each condition in each experiment. Results for each experiment are expressed as the means  $\pm$  S.D. of duplicate cultures, and all results described are representative of at least three separate experiments. ANOVA for three or more unpaired groups or Student's *t* test for two unpaired groups was used, and *P* < 0.05 was considered significant. The results are expressed as means  $\pm$  S.E.M. where the results of multiple experiments are pooled.

## RESULTS

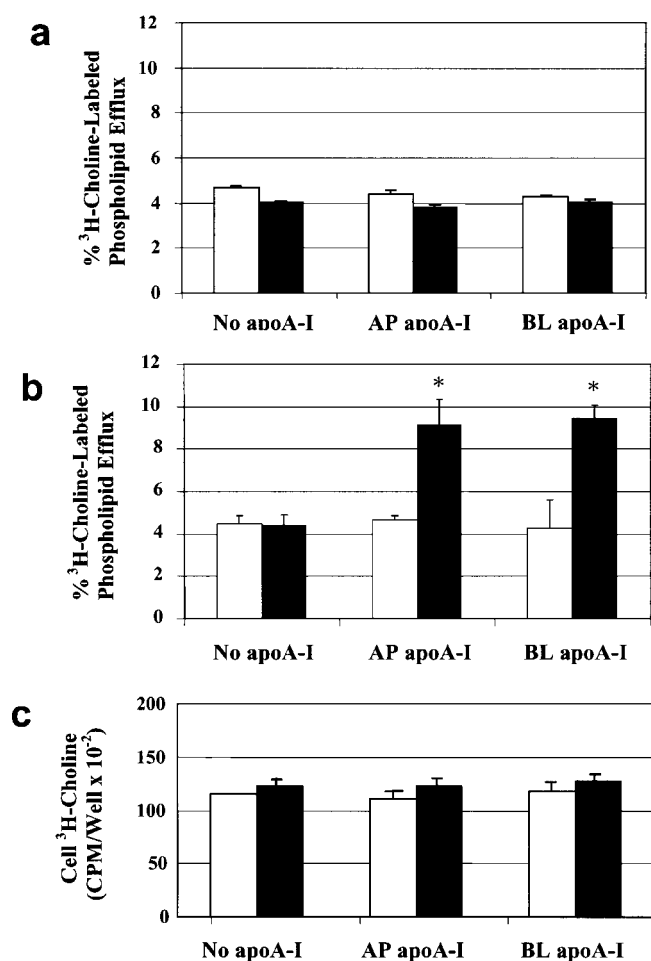
### Cholesterol efflux in GBEC is consistent with an ABCA1-mediated pathway

We adapted the cholesterol-efflux assay used previously on macrophages and fibroblasts to determine functional expression of ABCA1 [17]. In order to allow separate access to the AP and BL compartments, the cholesterol-efflux assay was performed on cells cultured on porous membrane supports (Transwells). Cholesterol loading and radiolabelling of GBEC were performed by



**Figure 1** Cholesterol efflux in GBEC

Cells were cultured, cholesterol-loaded with model bile and efflux assays performed with or without 8-Br-cAMP and with or without AP or BL apoA-I. Results are shown as percentage efflux, or as c.p.m./well  $\times 10^{-2}$  for the cellular-labelling results. (a) AP cholesterol efflux; (b) BL cholesterol efflux; (c) cellular cholesterol labelling. Open bars, no 8-Br-cAMP treatment; closed bars, 8-Br-cAMP treatment. Values are the means  $\pm$  S.E.M. from quadruplicate determinations, representative of five experiments. \**P* < 0.001, versus no cAMP or no apoA-I.



**Figure 2** Phospholipid efflux in GBEC

Assays were performed under the same conditions as for the cholesterol-efflux assays except that [ $^3\text{H}$ ]choline was used instead of [ $^{14}\text{C}$ ]cholesterol for cellular labelling. Results are shown as percentage efflux, or as c.p.m./well  $\times 10^{-2}$  for the cellular-labelling results. (a) AP phospholipid efflux; (b) BL phospholipid efflux; (c) cellular phospholipid labelling. Open bars, no 8-Br-cAMP treatment; closed bars, 8-Br-cAMP treatment. Values are the means  $\pm$  S.D. from triplicate determinations, representative of three experiments. \* $P < 0.001$ , versus no cAMP or no apoA-I.

incubating radiolabelled cholesterol with model bile containing supersaturating concentrations of cholesterol (cholesterol saturation index, 1.5) and adding the model bile to the AP surface of cells. Recrystallized cholesterol was used in preparing model bile to minimize the presence of oxysterols. Following washing, cholesterol efflux was measured under baseline conditions, and compared with efflux demonstrated following the addition of 8-Br-cAMP. De-lipidated human apoA-I was used as the cholesterol acceptor. The cholesterol efflux that is specifically elicited by cAMP and apoA-I is consistent with an ABCA1-mediated pathway [17,19]. This protocol gave specific data regarding the polarity of cholesterol efflux, and enabled examination of differences in cholesterol efflux with AP and BL addition of apoA-I.

Results of the cholesterol-efflux assays are shown in Figure 1. There was no significant cholesterol efflux into the AP compartment in the absence or presence of 8-Br-cAMP when apoA-I was omitted (Figure 1a). ApoA-I added to the AP compartment also did not elicit significant AP cholesterol efflux, with or

without 8-Br-cAMP (Figure 1a). Likewise, apoA-I added to the BL compartment did not elicit significant AP cholesterol efflux (Figure 1a). Significant cholesterol efflux was observed, however, into the BL compartment with BL apoA-I added in the presence of 8-Br-cAMP ( $P < 0.001$ ; Figure 1b). These results were not due to differential loading of cholesterol from model bile, as under all conditions tested cellular labelling with [ $^{14}\text{C}$ ]cholesterol did not differ significantly (Figure 1c). Surprisingly, BL cholesterol efflux was also elicited when apoA-I was supplied to the AP compartment in the presence of 8-Br-cAMP (Figure 1b). Therefore, both AP and BL apoA-I elicited BL cholesterol efflux, suggesting that the BL plasma membrane is the predominant site of functional ABCA1 expression. In other words, although cholesterol efflux occurred almost exclusively into the BL compartment, consistent with ABCA1 expression on the BL membrane, addition of apoA-I to either compartment stimulated this process.

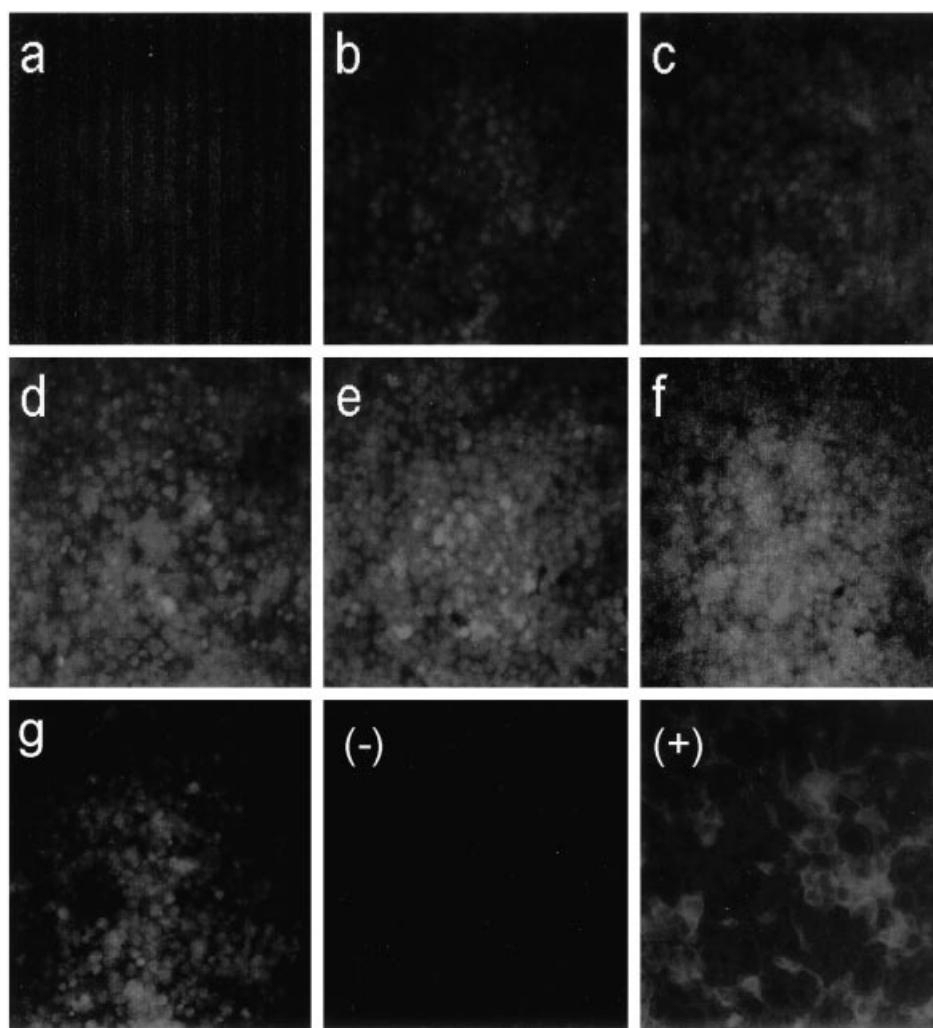
#### Phospholipid efflux in GBEC is consistent with an ABCA1-mediated pathway

Analogous to cholesterol efflux, phospholipid efflux was determined following cellular labelling with [ $^3\text{H}$ ]choline and model bile containing cholesterol for 48 h before addition of AP or BL apoA-I in the absence or presence of 8-Br-cAMP. ABCA1 effluxes both cholesterol and phospholipid, with apoA-I as the most commonly used acceptor [15]. There was no significant AP efflux of phospholipid following apoA-I addition to either the AP or BL side with or without 8-Br-cAMP (Figure 2a). In addition, there was no significant apoA-I-induced BL efflux of phospholipid in the absence of 8-Br-cAMP (Figure 2b). Predominantly BL phospholipid efflux was observed in the presence of 8-Br-cAMP, regardless of whether apoA-I was added to the AP or BL side (Figure 2b). Differential cellular labelling with [ $^3\text{H}$ ]choline did not account for these results (Figure 2c). Phospholipid efflux therefore paralleled cholesterol efflux in GBEC, and again showed the interesting finding that BL phospholipid efflux was stimulated whether apoA-I was supplied to the AP or the BL side.

#### Detection of ABCA1 in GBEC

Immunofluorescence microscopy and Western blotting were performed using a rabbit polyclonal antibody made against a peptide derived from the human C-terminal ABCA1 sequence [11]. This antibody is specific for ABCA1, as demonstrated previously in immunoblots [11,27]. GBEC were cultured under control conditions or cholesterol-loaded with model bile (with or without 8-Br-cAMP). In immunofluorescence microscopy studies (Figure 3), ABCA1 expression increased following cellular cholesterol loading with model bile compared with control conditions (no treatment with either model bile or 8-Br-cAMP; compare Figure 3e with Figure 3c). ABCA1 expression was increased further following treatment with both model bile and 8-Br-cAMP, compared with cells without treatment (Figure 3f compared with Figures 3c–3e). 8-Br-cAMP treatment alone also showed increased ABCA1 expression compared with the control condition (compare Figure 3d with Figure 3c). Negative controls with no primary antibody or incubation with pre-immune serum did not show a higher signal compared with the control conditions (Figures 3a and 3b compared with Figure 3c). In addition, cells treated with both model bile and 8-Br-cAMP and incubated with pre-immune serum and secondary antibody did not show an increased signal (Figure 3g).

In order to better characterize the specificity of the ABCA1 antibody on immunofluorescence studies, we also performed



**Figure 3** Detection of ABCA1 in GBEC by immunofluorescence microscopy

The experiments were performed three times for each condition. (a) Secondary antibody only (negative control); (b) pre-immune serum and secondary antibody (negative control); (c) primary/secondary antibodies; (d) 8-Br-cAMP treatment and primary/secondary antibodies; (e) model bile treatment and primary/secondary antibodies; (f) model bile and 8-Br-cAMP treatment and primary/secondary antibodies; (g) model bile and 8-Br-cAMP treatment and pre-immune serum/secondary antibody (negative control). The panels marked + and - show immunofluorescence results on BHK cells with an inducible ABCA1-containing plasmid without (-) and with (+) 10 nM mifepristone treatment.

these studies on BHK cells that expressed ABCA1 under a mifepristone-inducible system [27]. As shown in Figure 3 (+ and - panels), treatment of BHK cells containing the ABCA1-inducible plasmid with mifepristone showed a prominent signal (designated +), whereas untreated cells (-) did not. Studies on BHK cells not containing the ABCA1 plasmid, and studies in which the ABCA1 antibody was omitted, showed only background levels of fluorescence (results not shown).

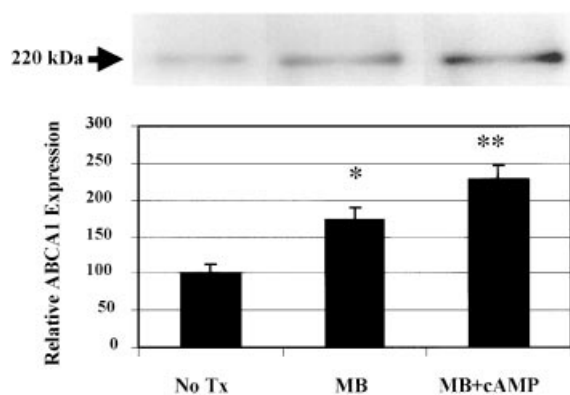
Western blotting was performed to complement the immunofluorescence studies. A specific band at 220 kDa, consistent with ABCA1, was detected (Figure 4). Densitometry showed that cellular cholesterol loading with model bile increased expression of ABCA1 (72% increase compared with control;  $P < 0.005$ ). 8-Br-cAMP treatment of cholesterol-loaded cells accentuated the expression of ABCA1 (130% increase compared with control;  $P < 0.005$ ).

ABCA1 expression was therefore increased when cells were loaded with cholesterol using model bile. This increase in ABCA1 expression was not correlated with increased cholesterol or

phospholipid efflux (see Figures 1b and 2b), suggesting a dissociation between ABCA1 expression levels and ABCA1 functional activity. Thus the added requirement for cAMP analogue for activation of ABCA1 function was evident in GBEC despite their cholesterol-loaded state. In addition, in cholesterol-loaded cells, 8-Br-cAMP treatment led not only to cholesterol and phospholipid efflux upon apoA-I exposure, but also to a further increase in ABCA1 expression (see Figures 3f and 4).

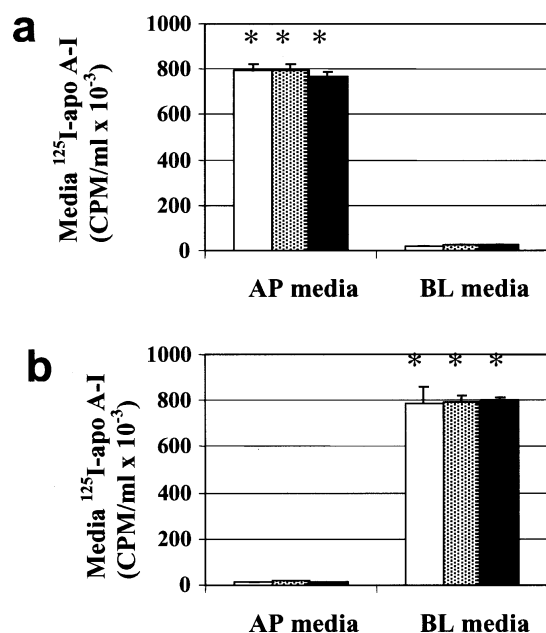
#### AP apoA-I does not induce BL cholesterol efflux via transcellular or paracellular passage in GBEC

The finding that AP apoA-I elicited BL cholesterol and phospholipid efflux suggested several possible mechanisms. One explanation is that apoA-I passes from the AP to the BL compartments due to leaky tight junctions between cells or trafficking of apoA-I through cells. To rule out these possibilities, the same conditions used for the lipid-efflux assay were kept in



**Figure 4** Detection of ABCA1 in GBEC by Western blotting

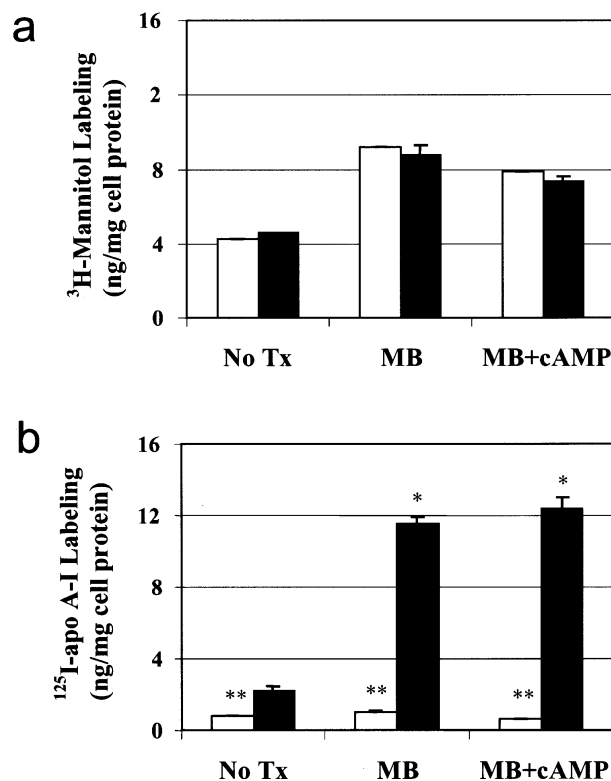
Results are expressed as the relative ABCA1 expression as percentage of control, with ABCA1 expression in untreated cells (No Tx) as the baseline. The values are the means  $\pm$  S.D. from duplicate determinations, representative of three experiments. \* $P < 0.005$ , versus no treatment (no Tx); \*\* $P < 0.005$ , versus no treatment and model bile (MB) treatment.



**Figure 5** Lack of significant transcellular or paracellular permeability of apoA-I in GBEC

Cells were cultured and treated as described in the Experimental section, except for incubation with SFM/BSA containing 10  $\mu$ g/ml <sup>125</sup>I-apoA-I and unlabelled apoA-I. Following 24 h of incubation, media from the AP and BL compartments were harvested, and direct  $\gamma$ -counting was performed. The same experiment was performed simultaneously with 2  $\mu$ Ci/ml [<sup>3</sup>H]mannitol as a control instead of <sup>125</sup>I-apoA-I. Results are shown as c.p.m./ml of media  $\times 10^{-3}$ . (a) <sup>125</sup>I-ApoA-I added to the AP side; (b) <sup>125</sup>I-apoA-I added to the BL side. Open bars, no treatment; hatched bars, model bile treatment; closed bars, model bile plus 8-Br-cAMP treatment. Values are the means  $\pm$  S.D. from duplicate determinations, representative of three experiments. \* $P < 0.001$  versus media from the opposite side.

experiments performed with <sup>125</sup>I-apoA-I or [<sup>3</sup>H]mannitol. The results showed no significant permeability of <sup>125</sup>I-apoA-I or [<sup>3</sup>H]mannitol from the AP to the BL compartments (or vice-versa; Figure 5). Therefore, AP apoA-I is unlikely to act as a BL acceptor for cholesterol or phospholipid that is effluxed by



**Figure 6** Preferential association of apoA-I with the BL membrane of GBEC

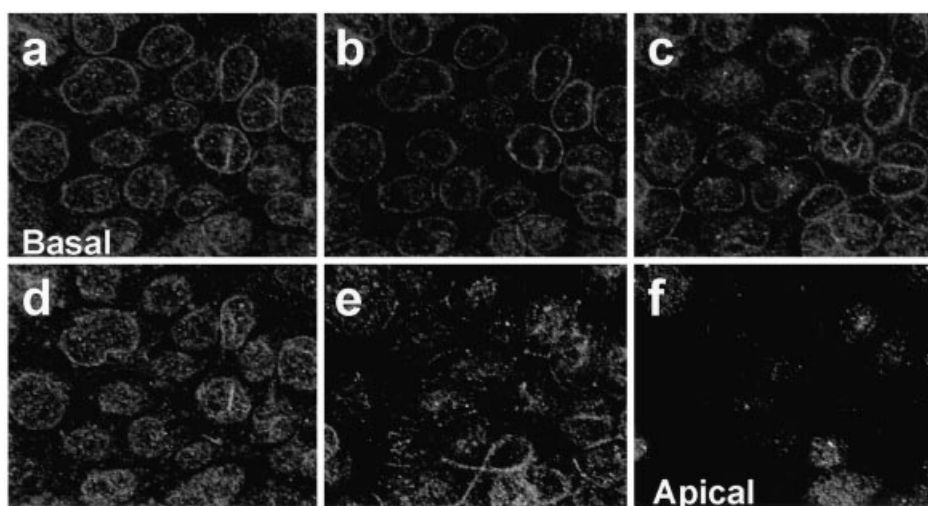
The experiment was performed with cells labelled with <sup>125</sup>I-apoA-I or [<sup>3</sup>H]mannitol. Results are shown as ng of apoA-I or ng of mannitol/mg of cell protein. (a) [<sup>3</sup>H]Mannitol cellular labelling; (b) <sup>125</sup>I-apoA-I cellular labelling. Open bars, AP application of radiolabel; closed bars, BL application of radiolabel. Values are the means  $\pm$  S.D. from duplicate determinations, representative of three experiments. \* $P < 0.001$ , versus all conditions for AP loading or no treatment (No Tx) condition for BL loading of <sup>125</sup>I-apoA-I; \*\* $P < 0.001$ , versus all conditions for AP loading of [<sup>3</sup>H]mannitol. MB, model bile.

ABCA1 in this *in vitro* system, because no paracellular or transcellular pathway exists for passage of this molecule.

#### ApoA-I associates preferentially with the BL membrane of GBEC

ApoA-I associates either directly with ABCA1, thereby facilitating ABCA1-mediated cholesterol efflux [19,29], or with the plasma membrane in the vicinity of ABCA1 [30]. We therefore asked whether conditions under which ABCA1 expression is increased lead to changes in apoA-I association with GBEC. In these experiments carried out at room temperature, apoA-I cellular association was a measure of apoA-I binding, uptake and degradation. <sup>125</sup>I-ApoA-I was added either apically or basolaterally to confluent cells, with or without model bile and 8-Br-cAMP. BL association of <sup>125</sup>I-apoA-I was greater than AP association. This difference was accentuated when model bile and 8-Br-cAMP were present (Figure 6b). In contrast, there was no significant difference between AP and BL association of [<sup>3</sup>H]mannitol (Figure 6a). These findings support the concept that the BL surface of GBEC associates preferentially with apoA-I under conditions that increase ABCA1 expression.

AP <sup>125</sup>I-apoA-I association was significantly less than AP [<sup>3</sup>H]mannitol association under each condition. As mannitol would be passively taken up by the cell, the lack of significant apoA-I association on the AP side also suggests that AP



**Figure 7** Localization of ABCA1 expression by confocal laser scanning immunofluorescence microscopy in GBEC

Images scanned in the  $xy$  plane from the basal (a) to the AP (f) aspect of cells following incubation with the ABCA1 antibody.

apoA-I did not undergo transcytosis from the AP to the BL side of the cell.

The dissociation between ABCA1 expression and function in cholesterol-loaded cells was supported by the  $^{125}\text{I}$ -apoA-I cell-association studies. Cellular association of  $^{125}\text{I}$ -apoA-I was increased to the BL membrane of cells following cholesterol loading with model bile (Figure 6b), conditions under which no increase in function of ABCA1 had been noted (Figures 1b and 2b). Therefore, the increase in BL membrane ABCA1 expression elicited by cholesterol loading of cells with model bile correlated with increased  $^{125}\text{I}$ -apoA-I cell association, but these events were not sufficient to permit cholesterol or phospholipid efflux in the absence of the cAMP analogue.

#### Localization of ABCA1 by confocal laser-scanning immunofluorescence microscopy in GBEC

Given the results of the cholesterol- and phospholipid-efflux assays and the results of the  $^{125}\text{I}$ -apoA-I cellular-association experiments, a predominantly BL localization of ABCA1 in GBEC appeared likely. In order to show more precise localization of ABCA1 in GBEC, we used confocal laser-scanning immunofluorescence microscopy. In images acquired in the  $xy$  plane (Figure 7), expression of ABCA1 appeared predominantly on the plasma membrane. A less-intense signal was noted in a punctate distribution intracellularly and on the sections taken at the most AP aspect of cells (Figure 7f). In these images, the most intense signal was in the BL plasma membrane, as these columnar epithelial cells have tight junctions near their AP surface, and as the cells contain a larger percentage of membrane in the BL plasma membrane than in the AP plasma membrane (compare Figures 7a–7d with Figures 7e and 7f).

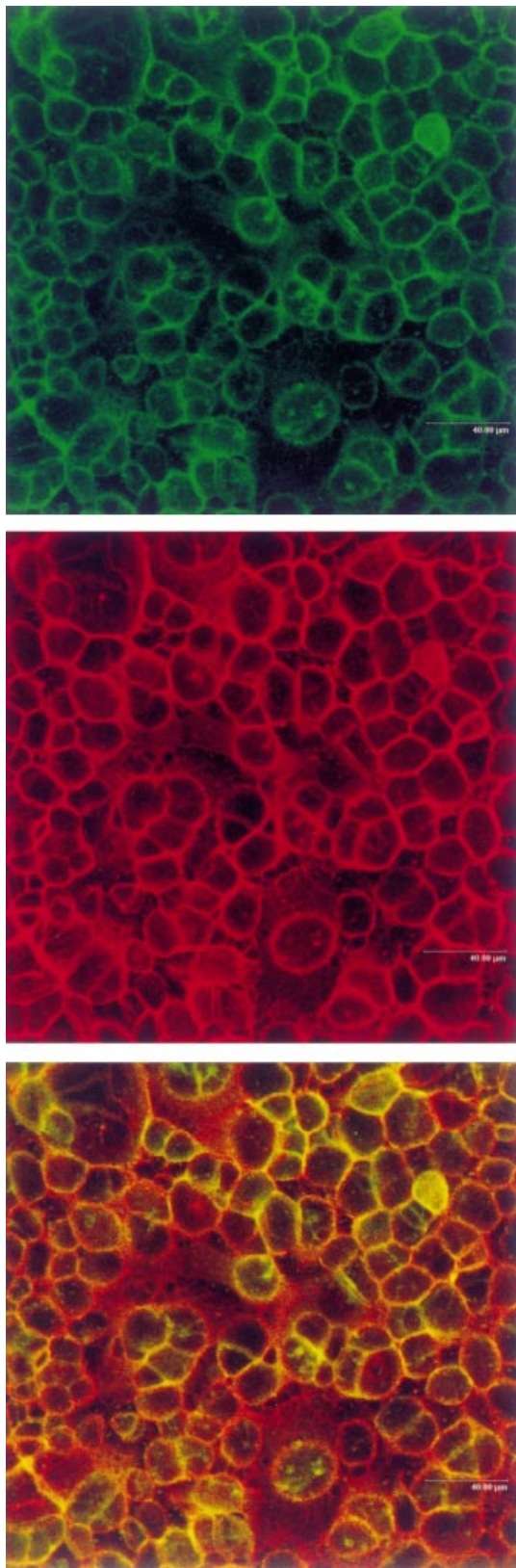
In order to better define the localization of ABCA1, confocal projection images on cells incubated with ABCA1 and the BL plasma membrane marker  $\text{Na}^+/\text{K}^+$ -ATPase were performed. The ABCA1 and the  $\text{Na}^+/\text{K}^+$ -ATPase signals showed co-localization on the overlay image (Figure 8). Intracellular ABCA1 signal was also noted, consistent with a report that ABCA1 shuttles from the plasma membrane to intracellular compartments in other cell types [31]. Negative controls using only the secondary antibodies did not show a fluorescence signal (results not shown).

#### LXR $\alpha$ /RXR ligands stimulate polarized cholesterol efflux in GBEC

Cholesterol efflux was also measured in response to ligands of the nuclear hormone receptors LXR $\alpha$  and RXR. During loading of cells with cholesterol using model bile, the LXR $\alpha$  ligand 22-(*R*)-hydroxycholesterol and the RXR ligand 9-*cis*-retinoic acid were added to confluent polarized cells. We used 10  $\mu\text{M}$  for each ligand, a concentration reported to elicit cholesterol efflux in macrophages and fibroblasts [23,24]. The LXR $\alpha$ /RXR ligands were added simultaneously to both the AP and BL surfaces in order to maximize cellular effects. We looked for evidence of polarized ABCA1-mediated cholesterol efflux following addition of the lipid acceptor apoA-I to either the AP or the BL compartments. Conversely, we looked for evidence of non-ABCA1-mediated cholesterol efflux in the absence of exogenous apoA-I.

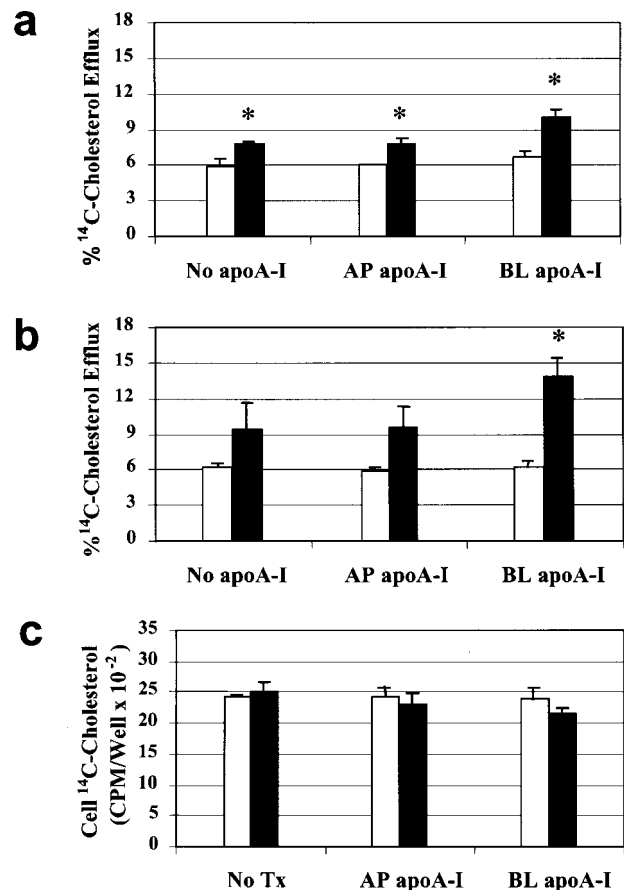
Cellular labelling with [ $^{14}\text{C}$ ]cholesterol under all conditions did not differ significantly, as shown in Figure 9(c). Treatment with ligands for LXR $\alpha$  and RXR elicited a significant AP efflux whether apoA-I was absent or supplied apically or basolaterally [ $P < 0.05$  for the comparison between the no-treatment group and the 22-(*R*)-hydroxycholesterol/9-*cis*-retinoic acid treatment groups; Figure 9a]. The results suggest that AP cholesterol efflux is not entirely dependent on exogenous apoA-I, implying that an ABCA1-mediated pathway is unlikely to play a major role. Stated another way, these results imply that non-ABCA1, LXR $\alpha$ /RXR-responsive pathways play a role in eliciting AP cholesterol efflux in GBEC.

The results for BL cholesterol efflux are shown in Figure 9(b). Without LXR $\alpha$ /RXR ligand treatment, there was no significant BL cholesterol efflux despite the presence of AP or BL apoA-I. Treatment with 9-*cis*-retinoic acid and 22-(*R*)-hydroxycholesterol led to a significant increase in BL cholesterol efflux when apoA-I was supplied basolaterally. Increased BL cholesterol efflux was also noted, however, with no apoA-I treatment, and with AP apoA-I treatment (although this did not reach statistical significance). This is similar to that noted with AP cholesterol efflux (Figure 9a). Overall, the findings suggest that a BL cholesterol-efflux pathway mediated by ABCA1 and responsive to LXR $\alpha$ /RXR ligands is present, which fits with the model of the presence of functional ABCA1 on the BL plasma membrane of GBEC. However, BL cholesterol efflux dependent on non-ABCA1-



**Figure 8** Co-localization of ABCA1 and  $\text{Na}^+/\text{K}^+$ -ATPase expression by confocal laser-scanning immunofluorescence microscopy in GBEC

Shown are  $xy$  projection images showing the same field scanned with fluorescence filters corresponding to the secondary antibodies for the ABCA1 antibody (top), the  $\text{Na}^+/\text{K}^+$ -ATPase antibody (middle) and the overlay image (bottom).



**Figure 9** LXR $\alpha$ /RXR-mediated cholesterol efflux in polarized GBEC

Results are shown as percentage efflux, or as c.p.m./well  $\times 10^{-2}$  for the cellular labelling results. (a) AP cholesterol efflux; (b) BL cholesterol efflux; (c) cellular cholesterol labelling. Open bars, no treatment; closed bars, 9-*cis*-retinoic acid and 22-(*R*)-hydroxycholesterol treatment. Values are the means  $\pm$  S.D. from triplicate determinations, representative of three experiments. \* $P < 0.05$ , versus the no-treatment group within each apoA-I treatment category.

mediated mechanisms, which are responsive to LXR $\alpha$ /RXR ligands, are also present.

## DISCUSSION

The major findings of the current work are: (i) cholesterol and phospholipid efflux consistent with an ABCA1-mediated pathway is present in cultured GBEC; (ii) this cholesterol and phospholipid efflux is predominantly BL in orientation in these polarized cells; (iii) AP application of apoA-I elicits BL cholesterol and phospholipid efflux in the absence of paracellular or transcellular passage of apoA-I following cAMP analogue treatment, and (iv) ligands for LXR $\alpha$  and RXR activate apoA-I-dependent and -independent cholesterol efflux to both AP and BL compartments. These findings provide insights into the mechanisms of cholesterol transport in GBEC.

This study contains several novel aspects. First is the use of a well-differentiated polarized epithelial cell-culture model to investigate ABCA1 function and localization. ABCA1 has been studied intensively in macrophages and fibroblasts, highlighting the importance of this transporter in the pathogenesis of atherosclerosis [15]. Issues regarding cell polarity are of lesser significance in macrophages and fibroblasts, but are of seminal importance in epithelial cells. For example, the seemingly paradoxical finding that ABCA1 and the HDL receptor scavenger-



receptor class B type I act in a futile cycle with respect to cholesterol uptake and efflux [32] may be due to the fact that those studies were performed in non-polarized cells. The canine GBEC-culture system, used extensively in our laboratory [5,26,28,33–35], provides a model well-suited to address cholesterol- and phospholipid-transport mechanisms in polarized epithelial cells.

An understanding of polarized cholesterol-transport pathways in epithelial cells has implications beyond the GB. ABCA1 is postulated to be expressed in enterocytes, and may be critical for effluxing dietary cholesterol [36,37]. ABCG5 and ABCG8 may act in concert with ABCA1 to regulate intake of dietary cholesterol and plant sterols [38]. Therefore, the mechanisms of action of ABCA1 and related ABC transporters in the intestinal epithelial cell are an important area of investigation. As the enterocyte and the GB epithelial cell share many transporters (such as the bile acid transporter [39], cystic fibrosis transmembrane conductance regulator ('CFTR') [40] and P-glycoprotein [41], among others), insights gained from the study of GBEC may have relevance to our understanding of cholesterol handling by enterocytes.

A second novel finding of this study is that AP apoA-I elicits BL cholesterol and phospholipid efflux following cAMP analogue treatment. ApoA-I is present in bile and possesses anti-nucleating properties [8,9]. Biliary apoA-I enhances mucosal-to-serosal transfer of cholesterol and phospholipid in cultured human GBEC [10], a finding that is supported by our data. The mechanism of the anti-nucleating action of biliary apoA-I and of the net mucosal-to-serosal transfer of cholesterol and phospholipid has not heretofore been defined. Our findings raise the possibility that apoA-I signals to the GB epithelial cell to increase cholesterol and phospholipid efflux via the BL plasma membrane. This may account for both the anti-nucleating and the cholesterol/phospholipid-transfer properties of biliary apoA-I. The mechanism is likely to be complex, as BL cholesterol/phospholipid efflux is dependent on the presence of the cAMP analogue. Further studies will be necessary to determine the mechanism involved in the AP apoA-I-mediated BL cholesterol/phospholipid-efflux phenomenon.

Cholesterol-loaded GBEC did not show significant apoA-I-mediated cholesterol or phospholipid efflux unless the cAMP analogue was also present. This was evident even when cholesterol loading alone induced ABCA1 expression and apoA-I cell association. This is in contrast with macrophages and fibroblasts, which show significant cholesterol and phospholipid efflux when cholesterol-loaded in the presence of a lipid acceptor, without the requirement for cAMP analogue [11,17,42]. One explanation for this discrepancy is that GBEC, which normally exist in a cholesterol-rich environment, may have a greater ability than other cell types to secrete cholesterol and phospholipids by ABCA1-independent mechanisms. In support of this idea are our results showing that addition of apoA-I enhanced efflux of these lipids less than 2-fold above background levels. Thus significant apoA-I-stimulated lipid efflux may not occur until ABCA1 expression exceeds a threshold, which in this case requires both cholesterol loading and 8-Br-cAMP treatment. Alternatively, a cAMP-sensitive lipid-transport process downstream from ABCA1 may be rate-limiting in GBEC. This appears unlikely, however, as we found that other ABCA1 inducers, such as ligands for LXR $\alpha$  and RXR [37], enhance apoA-I-mediated lipid efflux from GBEC in a manner similar to 8-Br-cAMP.

Ligands for the nuclear hormone receptors LXR $\alpha$  and RXR increase the expression and activity of ABCA1 [23,24]. The finding that LXR $\alpha$ /RXR ligand treatment activated cholesterol efflux in GBEC supports the concept that ABCA1 participates in

this pathway. The magnitude of efflux elicited by LXR $\alpha$ /RXR stimulation was higher than that elicited following treatment with the cAMP analogue. This suggests that transcriptional control of ABCA1 expression is a more potent inducer of cholesterol efflux than activation by cAMP analogue and cholesterol loading. In addition, the polarity of apoA-I-mediated efflux elicited by LXR $\alpha$ /RXR supports the concept that ABCA1 activity is localized predominantly on the BL plasma membrane. The data also suggest that cholesterol efflux by non-ABCA1-mediated pathways exists, and may account for some of the apically and basolaterally directed cholesterol efflux elicited by LXR $\alpha$ /RXR ligand stimulation.

The finding that AP and BL cholesterol efflux was elicited by the LXR $\alpha$ /RXR ligands in the absence of exogenous apoA-I, and the finding that AP apoA-I elicited BL cholesterol and phospholipid efflux following cAMP analogue treatment, implies that GBEC are capable of synthesizing and secreting endogenous apolipoproteins which could serve as cholesterol acceptors. Apolipoprotein E (apoE) is a candidate for this role. ApoE is found in a variety of cells and is involved in lipid transport [43]. In macrophages, endogenous apoE mediates cholesterol efflux via complex pathways [44]. ApoE mRNA levels are increased when GBEC are cholesterol-loaded (R. Kuver, unpublished work). Polarized GBEC express apoE on immunoblots, which increases upon cholesterol loading with model bile, and apoE is secreted by GBEC into the BL compartment following cholesterol loading (J. Lee and R. Kuver, unpublished work). Therefore, apoE, which acts as a lipid acceptor for ABCA1-mediated cholesterol efflux [45], could act as an endogenous lipid acceptor in GBEC. Given the complexities of sterol efflux mediated by endogenous and exogenous apoE in macrophages [44], and given the complexities of the relationship between apoE- and ABCA1-mediated lipid transport [46,47], additional studies are needed before apoE can be proven to be a major endogenously produced lipid acceptor for cholesterol and phospholipid efflux in GBEC.

The findings suggest that the GB participates in reverse cholesterol transport. We can speculate on the teleology of such a cholehepatic pathway for cholesterol. One possibility is that this is a defence mechanism that allows the GB to unload excessive amounts of absorbed cholesterol and phospholipid, the majority of which enters the cell passively [3]. This may be relevant for cholesterol gallstone formation, as inhibition of ABCA1-mediated cholesterol efflux by the pro-nucleating anionic peptide fraction/calcium-binding protein, which shares sequence homology with apoA-I, has been demonstrated [48]. The roles of other ABC transporters, nuclear hormone receptors, endogenous apolipoproteins and scavenger-receptor class B type I in mediating cholesterol transport in GBEC remain to be defined. Future studies will no doubt reveal the complexities involved in cholesterol transport in these cells.

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