1. Introduction

The Niemann–Pick type C1 and C2 proteins (NPC1 and NPC2) function within late endosomes and lysosomes to make cholesterol derived from hydrolysis of lipoprotein cholesterol esters or from membrane turnover available to the rest of the cell [12,2]. Previous and recent work has provided evidence that NPC2 binds cholesterol [3,4], and delivers it within the lysosomal lumen to NPC1 in the lysosomal membrane for subsequent egress from this compartment [5–7].

Abbreviations: ApoA-I, apolipoprotein A-1; ABCA1, ATP-binding cassette transporter A1; EGP, enhanced green fluorescent protein; HDL, high density lipoprotein; LDL, low density lipoprotein; LXr, liver X receptor; NPC, Niemann–Pick type C.

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Deficiency of either NPC2 or NPC1 therefore results in sequestration of excess unesterified cholesterol within late endosomes/lysosomes, impaired delivery of cholesterol to the endoplasmic reticulum for esterification or regulation of cholesterol homeostatic genes including HMG-CoA reductase and the LDL receptor, and impaired generation of oxysterols [8–10]. Deficiency of activity of either protein leads to a similar and frequently fatal neurodegenerative and hepatic disorder, Niemann–Pick disease type C [1].

We previously reported impaired cholesterol-dependent regulation of the key protein regulating new high density lipoprotein (HDL) formation, the ATP-binding cassette transporter A1 (ABCA1), in patients with NPC disease [11]. This finding is consistent with impaired oxysterol generation, the key regulator of liver X receptor (LXR)-dependent activation of ABCA1 expression, in both NPC1−/− and NPC2−/− cells [10], and provides a likely mechanism for the low plasma HDL-C seen in the majority of NPC disease patients [11,12]. We subsequently found that treatment of NPC1−/− cells

ABCA1-dependent mobilization of lysosomal cholesterol requires functional Niemann–Pick C2 but not Niemann–Pick C1 protein☆

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ABSTRACT

Niemann–Pick disease type C (NPC) is caused by mutations leading to loss of function of NPC1 or NPC2 proteins, resulting in accumulation of unesterified cholesterol in late endosomes and lysosomes. We previously reported that expression of the ATP-binding cassette transporter A1 (ABCA1) is impaired in human NPC1−/− fibroblasts, resulting in reduced HDL particle formation and providing a mechanism for the reduced plasma HDL cholesterol seen in the majority of NPC1 patients. We also found that treatment of NPC1−/− fibroblasts with an agonist of liver X-receptor corrects ABCA1 expression and HDL formation and reduces lysosomal cholesterol accumulation. We have confirmed that ABCA1 expression is also reduced in NPC2−/− cells, and found that α-HDL particle formation is impaired in these cells. To determine whether selective up-regulation of ABCA1 can correct lysosomal cholesterol accumulation in NPC disease cells and HDL particle formation, we produced and infected NPC1−/− and NPC2−/− fibroblasts with an adenovirus expressing full-length ABCA1 and enhanced green fluorescent protein (AdABCA1-EGFP). ABCA1-EGFP expression in NPC1−/− fibroblasts resulted in normalization of cholesterol efflux to apolipoprotein A-I (apoA-I) and α-HDL particle formation, plus a marked reduction in filipin staining of unesterified cholesterol in late endosomes/lysosomes. In contrast, AdABCA1-EGFP treatment of NPC2−/− fibroblasts to normalize ABCA1 expression had no effect on cholesterol efflux to apoA-I or accumulation of excess cholesterol in lysosomes, and only partially corrected α-HDL formation by these cells. These results suggest that correction of ABCA1 expression can bypass the mutation of NPC1 but not NPC2 to mobilize excess cholesterol from late endosomes and lysosomes in NPC disease cells. Expression of ABCA1-EGFP in NPC1−/− cells increased cholesterol available for esterification and reduced levels of HMG-CoA reductase protein, effects that were abrogated by co-incubation with apoA-I. A model can be generated in which ABCA1 is able to mobilize cholesterol, to join the intracellular regulatory pool or to be effluxed for HDL particle formation, either directly or indirectly from the lysosomal membrane, but not from the lysosomal lumen. This article is part of a Special Issue entitled Advances in High Density Lipoprotein Formation and Metabolism: A Tribute to John F. Oram (1945–2010).

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with a nonoxysterol agonist of LXR corrected ABCA1 and ABCG1 expression, lipid efflux to apoA-I, and HDL formation, and could bypass the NPC1 mutation to reduce accumulation of late endosome/lysosome cholesterol [13]. In the present studies we aimed to determine whether an increase in ABCA1 expression alone has a similar effect in NPC1−/− cells, as well as the effect in NPC2−/− cells. We confirmed that ABCA1 expression is also impaired in NPC2−/− cells. Overexpression of ABCA1 using adenoviral gene delivery was able to correct cholesterol efflux to apoAI and HDL particle formation, and to markedly reduce lysosomal cholesterol accumulation in NPC1− but not NPC2-deficient cells. In addition, ABCA1 expression in the absence of apoA-I increased the intracellular cholesterol pool regulating cholesteryl ester formation and reduced HMG-CoA reductase protein in NPC1−/− cells. We conclude that ABCA1 can bypass mutations in NPC1 to mobilize cholesterol either directly or indirectly from the lysosomal membrane, but cannot overcome mutations in NPC2 to mobilize cholesterol from the lysosomal lumen.

2. Materials and methods

2.1. Materials

Fatty acid-free bovine serum albumin (hereafter just referred to as albumin) and filipin were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin, Dulbecco’s modified Eagle’s medium (DMEM) and lipoprotein-deficient serum (LPDS) were from HyClone (South Logan, UT). [cholesteryl-1,2,6,7,14H]cholesteryl linoleate (84 Ci/mmol) was from PerkinElmer Life Sciences (Boston, MA).

2.2. Cloning of ABCA1

Expression pcDNA3.1 plasmid encoding full-length murine ABCA1, a kind gift from Dr. Nan Wang, Columbia University [14], was amplified by PCR with the primers (5′-AGA GAG AGT CTC GAG TCT AGA CCA CCA TGG CTT GTT CGC CTC ACT TA-3′ containing Xhol and Xhol sites and 5′-GAG AGT CCT CGG TGG TAC TATA CTT CTT TAT CCC CAC TCT ATG-3′ containing Sac II site) and pfu polymerase (Invitrogen, Burlington, Canada). The reaction mixture was prepared in duplicate and contained 10 ng of cDNA and 12.5 pmol of each primer. The reaction mixture was preheated at 85 °C for 5 min prior to the addition of pfu polymerase, followed by initial denaturation at 95 °C for 2 min, 25 times cycling of CDNAS at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 8 min, and final extension at 72 °C for 10 min. The products were pooled together, purified by QIAquick PCR Purification Kit (Invitrogen), cloned into pEGFP-N1 vector (Clontech, Palo Alto, CA) in frame with EGFP gene using XhoI and Sac II restriction endonucleases, and the construct confirmed by sequencing.

2.3. Recombinant adenovirus preparation

We used E1/E3 deleted replication defective human adenovirus serotype 5 Adeno-x Expression System 1 (Clontech) to generate recombinant ABCA1-EGFP-expressing adenovirus. Generation of adenovirus was performed according to the protocol described by the manufacturer with some modifications. ABCA1-EGFP cDNA construct cloned into pShuttle2 vector yields an expression cassette of 8.3 kb, which exceeds the manufacturer’s suggested insertion capacity of Adeno-x viral DNA (i.e., 8.0 kb). Because of this, we modified the transfer region of pShuttle2 vector to a smaller region that is 320 bases less than the original pShuttle2 vector by PCR with primers (5′-ACA CAC AGA TCT TTA CAT AAC TTA CCG TAT AGT G-3′ containing Bgl II site and 5′-ACA CAC AGA TCT GCA GAT GAG TTT GGA-3′ containing Bgl II site) and pfu polymerase (Fig. 1). The PCR reaction mixture contained 10 ng of pShuttle2 and 12.5 pmol of each primer; the reaction conditions with 30 cycles were identical to those described above for cloning of ABCA1. The PCR product was purified by QIAquick PCR Purification Kit, and cloned into Bgl II restriction endonuclease digested pShuttle2 vector that had its Bgl II restriction endonuclease digestion sites dephosphorylated with Shrimp alkaline phosphatase (Fermentas, Burlington, Canada). ABCA1-EGFP construct was subcloned from pEGFP-N1 plasmid into the Not I and Xba I sites of modified pShuttle2 to construct the transfer plasmid pShuttle2-ABCA1-EGFP. EGFP was subcloned into Nhe I and Not I sites of modified pShuttle2 to make pShuttle2-EGFP (control). The expression cassette from pShuttle2-ABCA1-EGFP or pShuttle2-EGFP was ligated with I-Ceu and Pst-I cleaved Adenox viral DNA and subsequently transformed into Max Efficiency DH5α competent cells (Invitrogen). Recombinants were selected by ampicillin resistance and confirmed by I-Ceu and Pst-I restriction endonuclease analysis, and named Ad-ABCA1-EGFP and Ad-EGFP, respectively (Fig. 2A). The recombinant plasmid was linearized with Pac I restriction endonuclease, and 10 μg of the plasmid transfected into subconfluent HEK293 cells in 60 mm dish using calcium phosphate (Clontech) according to the manufacturer’s instructions. Recombinant adenoviruses obtained after 18 days post-transfection were amplified in HEK293 cells and purified by centrifugation on cesium chloride gradient.

2.4. Preparation of lipoproteins and ApoA-I

HDL (d = 1.07–1.21 g/ml) and LDL (d = 1.019–1.063 g/ml) were isolated from pooled plasma of healthy volunteers by ultracentrifugation [15]. ApoA-I was purified from delipidated HDL on Q-Sepharose Fast Flow column using the method of Yokoyama et al. [16]. Radiolabeling of LDL with [1,2,6,7,14H]cholesteryl linoleate to a specific activity of 16–44 cpm/ng of LDL protein was performed as described by Sattler and Stocker [17].

2.5. Cell culture

Normal human skin fibroblasts (wild type, CRL-2076) were purchased from the American Type Culture Collection. Human NPC1 null mutant fibroblasts line 93.41 (NPC1−/−, hereafter referred to as NPC1 cells) were provided by Dr. W. Garver (Dept. of Pediatrics, The University of Arizona, Arizona, USA). NPC2−/− mutant human skin fibroblasts (hereafter referred to as NPC2 cells, NIH 99.04) were provided by Dr. D. Ory (Center for Cardiovascular Research, Washington University School of Medicine, St. Louis, USA). Wild type and NPC2 fibroblasts were plated at 30,000 cells/16-mm well or 80,000 cells/35-mm well, and NPC1 fibroblasts were plated at 40,000 cells/16-mm well or 100,000 cells/35-mm well. The cells were grown to 90% confluence in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. The medium was then replaced with DMEM containing 10% LPDS for 24 h followed by adenoviral infection. The wild type and NPC1 fibroblasts were infected with recombinant adenovirus at multiplicity of infection (MOI) of 50 and NPC2 fibroblasts were infected at MOI of 100 in Opti-mem media without serum for 4 h; these MOIs were found in control experiments to provide equivalent expression of ABCA1. After the initial infection period, an equal volume of DMEM/20% LPDS plus antibiotics was added to the inoculum and the cells cultured for an additional 44 h prior to cholesterol loading.

2.6. Cholesterol efflux

Following infection, fibroblasts in 16-mm wells were labeled with LDL-derived cholesterol by incubation with DMEM containing 1 mg/ml albumin (DMEM/albumin) plus 50 μg/ml [1,2,6,7,14H]cholesteryl linoleate-labeled LDL protein for 24 h. Cells were washed twice with DMEM and equilibrated for 24 h in DMEM/albumin. After equilibration, the cells were rinsed twice with DMEM and were incubated with either DMEM/albumin alone or DMEM/albumin plus 10 μg/ml

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human apolipoprotein A-I (apoA-I) for 24 h. The media were collected and the cells washed three times with ice-cold PBS, and solubilized in 0.1 N NaOH. Efflux media were centrifuged at 2000× g for 5 min at 4 °C to remove cell debris. Radioactivity in media was determined by liquid scintillation counting. Protein concentration of cell lysates was determined using bovine serum albumin as standard [18].

2.7. Cholesterol esterification by ACAT

To assess the effect of ABCA1 expression on the pool of intracellular cholesterol regulating cholesterol esterification by acyl-CoA:cholesterol acyltransferase (ACAT), wild type and NPC1 fibroblasts were grown in 16-mm wells and the NPC1 cells infected with Ad-ABCA1-EGFP.

Fig. 1. Modification of pShuttle2 vector. Reduction of the transfer region of pShuttle2 by 330 bases was performed by introducing a new Bgl II site at position 330 by PCR. A forward primer (5′-ACA CAC AGA TCT TTA CAT ACG TCG TAA ATG G-3′) containing Bgl II site designed to match the initial sequence of Pcmv promoter and a reverse primer (5′-ACA CAC AGA TCT GCA GAC TTT GGA-3′) matching Bgl II site at position 1121 and pfu polymerase were used by PCR to generate a smaller fragment (Bgl II 330–Bgl II 1121) of the transfer region of pShuttle2. PCR conditions are described in the Materials and methods. The truncated fragment was cloned into Shrimp alkaline phosphatase dephosphorylated Bgl II sites of pShuttle2.

Fig. 2. Generation of recombinant adenoviral vectors expressing ABCA1-EGFP fusion protein and EGFP. Adenoviral vectors expressing ABCA1-EGFP fusion protein and EGFP (control) were prepared as described in the Materials and methods. (A) The expression of ABCA1-EGFP or EGFP gene was under the upstream control of the human cytomegalovirus immediate-early gene promoter (Pcmv IE) and the downstream control of the SV40 polyadenylation signal. (B) The adenoviral vectors were digested with Pci I to expose the inverted terminal repeats (ITRs) located at both ends of the adenoviral genome. Lane 1, 1 Kb plus DNA size marker; lane 2, Ad-EGFP; lane 3, Ad-ABCA1-EGFP. (C) Microscopic appearance of EGFP expression in HEK293 cells infected at a multiplicity of infection (MOI) of 2 with either Ad-EGFP (a) or Ad-ABCA1-EGFP (b) for 1 h and cultured for an additional 20 h. Bar = 20 μm. (D) Immunoblot analysis of the infected HEK293 cells confirmed EGFP-tagged ABCA1 protein expression. Nitrocellulose membrane was probed with ABCA1 antibody and then reprobed with GFP antibody.

EGFP as described above. The cells were then incubated in DMEM/albumin containing 50 μg/ml LDL for 24 h. Cells were rinsed twice with DMEM and then equilibrated for 24 h in DMEM/albumin. Following equilibration, the cells were incubated with DMEM/albumin ± 10 μg/ml apoA-I for 24 h, rinsed with DMEM, and incubated for 1 h at 37 °C with DMEM containing 9 μM [14C]oleate bound to 3 μM BSA [19]. Cells were then placed on ice, washed twice with cold PBS-BSA and twice with cold PBS, and total lipids extracted using hexane/2-propanol (3:2 v/v) as described [20]. Lipids were separated by thin-layer chromatography on PE SIL G plastic-backed plates (Whatman) developed in hexane/diethyl ether/acetic acid (130:40:1.5 v/v). Lipid spots were detected by staining in I2 vapor and comigration with standards. After allowing I2 stain to evaporate, cholesteryl ester spots were taken for determination of radioactivity. The cells were solubilized with 0.1 N NaOH and used for determination of protein. ACAT activity was determined by normalizing the amount of cholesteryl ester formed to the corresponding cellular protein.

2.8. Filipin staining

Fibroblasts were grown on coverslips in 35-mm wells and infected with recombinant adenovirus. The cells were loaded with non-labeled LDL and incubated with apoA-I as described above. Cells were then fixed and stained with filipin as described [13].

2.9. Immunoblot analysis

Cells in 35-mm wells were lysed with 100 μl of radioimmunoprecipitation assay (RIPA) buffer [PBS, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% nonidet P-40 (NP-40), pH 7.4] supplemented with Complete Protease Inhibitor Cocktail Tablet (Roche Diagnostics, Indianapolis, IN) for 1 h at 4 °C. Sixty microliters of total lysate proteins were separated by SDS-PAGE on 5–15% gradient gels, and transferred onto nitrocellulose membranes. Immunoblotting was performed using rabbit anti-human ABCA1 (1:1000 dilution, Novus Biologicals, Littleton, CO), rabbit anti-GFP (which also recognizes EGFP; 1:2000 dilution, Molecular Probes, Eugene, OR), rabbit anti-HMG-CoA reductase (1:500 dilution, Upstate Cell Signaling Solutions, Lake Placid, NY) and rabbit anti-β-actin (Abcam, Cambridge, MA) as primary antibodies, with goat anti-rabbit IgG horse-radish peroxidase-conjugated secondary antibody (1:5000 dilution, Sigma). Proteins were visualized by using enhanced chemiluminescence reagents from Pierce (Rockford, IL).

2.10. Two-dimensional gel electrophoresis of HDL particles

Media with apoA-I-containing particles generated by cells in 35-mm wells were analyzed by non-denaturing 2-dimensional gel electrophoresis as previously described [13], substituting goat anti-rabbit IgG horse-radish peroxidase-conjugated secondary antibody (1:5000 dilution, Sigma) and enhanced chemiluminescence reagents (Pierce) for [125I]-labeled donkey anti-rabbit antibody (Amersham Biosciences) and autoradiography.

2.11. Statistical analysis

Results for Figs. 3B, 5 and 8 were analyzed using GraphPad Prism version 5.0 and are presented as the mean ± S.D. Significant differences between experimental groups were determined using the Student’s t test.

3. Results

3.1. Generation of adenoviral vector expressing ABCA1-EGFP fusion protein

To determine whether our previous results showing reduced lysosomal cholesterol accumulation and enhanced HDL formation by NPC1-deficient fibroblasts treated with an LXR agonist [13] could be reproduced by upregulating ABCA1 alone, we required an efficient method of overexpressing ABCA1 in these cells. Adenoviral vectors have been vehicles of choice for gene expression in skin fibroblasts, since nonviral vector-mediated gene transfer using

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**Fig. 3.** Diminished ABCA1 expression in NPC1 and NPC2 fibroblasts. (A) Wild type (WT), NPC1 and NPC2 fibroblasts were grown to 60% confluence in DMEM/10% FBS and then cultured in DMEM/10% LPDS to confluence. The cells were incubated in DMEM/albumin in the absence or presence of LDL (50 μg/ml) for 24 h, and then equilibrated in DMEM/albumin for 24 h prior to determination of ABCA1 by immunoblotting. ABCA1 was detected using 60 μg of total cell protein. Numerical values represent the ratio of ABCA1 to β-actin intensity, normalized to the ratio in non-LDL loaded wild type fibroblasts set as one. Results are representative of three experiments with similar results. (B) Average ABCA1 protein levels as determined by immunoblotting in cells incubated in the absence (−) or presence (+) of LDL, relative to non-LDL-loaded wild type cells. Results are averages ± S.D. for 3 experiments. * significantly lower than non-LDL-loaded wild type fibroblasts, p < 0.05. ** significantly lower than LDL-loaded wild type fibroblasts, p < 0.0001.
various transfection methods is very inefficient in these cells. We therefore generated adenovirus expressing ABCA1-EGFP fusion protein or control EGFP alone using the Clontech Adeno-x expression system 1. Based on the manufacturer’s advice that this system has an insertion capacity of 8.0 kb (http://www.clontech.com/xxclt_ibcGetAttachment.jsp?cItemId=17524&minisite=10020), we modified the transfer region of pShuttle2 (Fig. 1) and used that to generate adenoviral vectors Ad-ABCA1-EGFP and Ad-EGFP (Fig. 2A). Digestion of the adenoviral vectors with Pac I restriction endonuclease resulted in the release of a larger fragment and a smaller fragment of 3.0 kb (Fig. 2B), consistent with a previous report [21]. Initial evaluation of the newly generated Ad-ABCA1-EGFP and Ad-EGFP-infected HEK293 cells by fluorescence microscopy showed bright green fluorescence in HEK293 cells (Fig. 2C), indicating the infectivity of the viruses and GFP expression in the cells. Immunoblot analysis confirmed the expression of ABCA1-EGFP fusion protein by Ad-ABCA1-EGFP but not Ad-EGFP (Fig. 2D). To the best of our knowledge, there is no prior demonstration of a single adenovirus expressing ABCA1-EGFP fusion protein.

3.2. ABCA1 expression in NPC1 and NPC2 human fibroblasts and increase with ABCA1-EGFP fusion protein expression

We previously demonstrated low basal and cholesterol-stimulated ABCA1 protein levels in human NPC1 fibroblasts [11], which were corrected by treatment of the cells with an exogenous LXR ligand TO-901317 [13]. The inability of NPC2-deficient fibroblasts to correctly deliver cholesterol to the lysosomal membrane for egress and stimulation of oxysterol generation suggests NPC2-deficient cells should also exhibit impaired ABCA1 expression. We confirmed our previous finding of impaired basal and LDL-stimulated expression of ABCA1 in NPC1-deficient fibroblasts, and also demonstrate this defect in NPC2-deficient cells (Figs. 3 and 4B). Infection of wild type, NPC1 and NPC2 fibroblasts with Ad-EGFP resulted in a diffuse pattern of EGFP fluorescence, and a punctuate pattern of fluorescence upon infection with Ad-ABCA1-EGFP (Fig. 4A), as previously reported in fibroblasts and other cell types using transfection or adenoviral infection [22–25]. Expression of ABCA1-EGFP fusion protein was seen in all cell types following infection with Ad-ABCA1-EGFP (Fig. 4B).

3.3. Enhanced cholesterol efflux in ABCA1-EGFP-expressing NPC1 but not NPC2 fibroblasts

NPC1 and NPC2 cells were infected with Ad-ABCA1-EGFP and then incubated with LDL labeled with [3H]cholesteryl linoleate prior to incubation with apoA-I to assess cholesterol efflux. Consistent with impaired ABCA1 expression, NPC1 and NPC2 fibroblasts both showed approximately 50% less efflux of LDL-derived cholesterol to apoA-I when compared to wild type fibroblasts (Fig. 5). Overexpression of ABCA1 in NPC1 cells resulted in correction of apoA-I-mediated efflux of LDL-derived cholesterol to a level similar to wild type fibroblasts infected with AdABCA1-EGFP. Infection of NPC2 cells with this vector,
two experiments with similar results. +, signi
cantly higher than non-infected wild type fibroblasts, p<0.01. *, significantly higher than non-infected wild type fibroblasts, p<0.05.

however, resulted in no increase in apoA-I-dependent efflux of cholesterol from LDL. These results suggest correction of ABCA1 expression is able to overcome the defect in lysosomal cholesterol mobilization in NPC1- but not NPC2-deficient cells.

3.4. Normalization of HDL particle formation by NPC1 but not NPC2 cells expressing ABCA1-EGFP

We previously reported impaired α-HDL particle formation by NPC1 fibroblasts incubated with apoA-I, consistent with a role of ABCA1 in generating α-HDL, and correction of formation of α-HDL particles by NPC1 cells following incubation with exogenous LXR agonist [13]. To determine the role of correcting ABCA1 expression alone on HDL particle formation by NPC1 and NPC2 cells, we assessed the HDL particles formed in apoA-I-containing medium by cells before and after infection with Ad-ABCA1-EGFP using two-dimensional gel electrophoresis. The medium of NPC1 cells showed near absence α-HDL particles, consistent with our previous report [13], as did NPC2 cell medium (Fig. 6A). Over-expression of ABCA1 by the adenoviral vector increased α-HDL particle formation by NPC1 fibroblasts to a similar extent as in wild type cells (Fig. 6B). α-HDL particle formation by NPC2 cells was increased only modestly by this treatment, likely because of increased efflux of non-lysosomal rather than lysosomal cholesterol by the increased ABCA1 protein. These results indicate further the importance of lysosomally-derived cholesterol in the generation of HDL particles by ABCA1.

3.5. Mobilization of late endosome/lysosome cholesterol by ABCA1-EGFP in NPC1 but not NPC2 fibroblasts

To more directly assess the effect of overexpression of ABCA1 on mobilization of cholesterol from late endosomes and lysosomes, NPC1 and NPC2 cells infected with control Ad-EGFP or Ad-ABCA1-EGFP were then loaded with LDL, incubated for 24 h in the absence or presence of 10 μg/ml apoA-I, and unesterified cholesterol remaining in the cells was detected using filipin. Incubation of non-infected or Ad-EGFP-infected NPC1 and NPC2 fibroblasts with albumin alone or albumin plus apoA-I resulted in no change in the intensity of filipin staining (Fig. 7). Expression of ABCA1-EGFP in NPC1 fibroblasts resulted in a marked decrease in filipin staining whether or not the cells were also incubated with apoA-I. Overexpression of ABCA1 in NPC2 cells, conversely, had no effect on accumulation of cholesterol in late endosomes/lysosomes in the absence or presence of apoA-I. These results further suggest that ABCA1 expression can overcome the defect in cholesterol mobilization from late endosomes/lysosomes in the absence or presence of apoA-I as a cholesterol (and phospholipid) acceptor.

3.6. Effect of ABCA1-EGFP expression on the regulatory pool of cholesterol in NPC1 fibroblasts

The impaired egress of cholesterol out of late endosomes and lysosomes in Niemann–Pick disease type C1 and C2 cells results in reduced esterification of cholesterol by acyl-coA:cholesterol acyltransferase

Fig. 5. ABCA1-EGFP expression increases LDL-derived cholesterol efflux to apoA-I in NPC1 but not NPC2 fibroblasts. Wild type (WT), NPC1 and NPC2 fibroblasts were infected with Ad-ABCA1-EGFP (indicated as +, ABCA1, open bars) as described in the Materials and methods. The cells were labeled with [3H]cholesteryl linoleate-labeled LDL, followed by equilibration and incubation with DMEM/albumin containing 10 μg/ml apoA-I for 24 h. Results are expressed as counts/min of [3H]cholesterol per mg of cell protein in the medium after subtraction of efflux to medium containing albumin alone. Values are the mean±S.D. of triplicate determinations and are representative of two experiments with similar results. +, significantly lower than non-infected wild type fibroblasts, p<0.01. *, significantly higher than non-infected wild type fibroblasts, p<0.05.

Fig. 6. Increased HDL particle formation in NPC1 fibroblasts by ABCA1-EGFP fusion protein. Media from non-infected (A) and Ad-ABCA1-EGFP-infected (B) wild type (WT), NPC1 and NPC2 fibroblasts that had been LDL-loaded, equilibrated and incubated with apoA-I as described in the Materials and methods were analyzed for apoA-I-containing HDL particles by two-dimensional gel electrophoresis. Results are representative of two experiments with similar results.

ACAT), increased expression of HMG-CoA reductase and de novo cholesterol synthesis, and impaired downregulation of LDL receptor expression in response to LDL loading when compared to normal cells [8–10,26]. To determine potential effects of increased mobilization of late endosome/lysosome cholesterol by overexpressing ABCA1 on the regulatory pool of cholesterol in NPC1 cells, we measured the degree of new cholesteryl ester formation and expression of HMG-CoA reductase in the absence or presence of apoA-I. NPC1 cells showed reduced cholesterol esterification in the absence of apoA-I, and no reduction in ACAT-accessible cholesterol following incubation with apoA-I when compared to normal cells (Fig. 8).

Overexpression of ABCA1 with Ad-ABCA1-EGFP in the absence of apoA-I resulted in increased cholesteryl ester formation, suggesting cholesterol mobilized from late endosomes/lysosomes in response to increased ABCA1 becomes available for esterification in the endoplasmic reticulum. This effect, however, was prevented by the addition of apoA-I. Consistent with these findings, Ad-ABCA1-EGFP infection of NPC1 cells reduced HMG-CoA reductase protein levels in the absence of apoA-I (Fig. 9). Addition of apoA-I removed this suppression of HMG-CoA reductase, again suggesting the increase in the regulatory pool of cholesterol induced by increasing ABCA1 was prevented by removal of this cholesterol by apoA-I.

Fig. 7. ABCA1-EGFP expression decreases late endosome/lysosome cholesterol in NPC1 but not NPC2 fibroblasts. Wild type (WT), NPC1 and NPC2 fibroblasts were infected with either Ad-EGFP or Ad-ABCA1-EGFP. The cells were then loaded with LDL for 24 h and incubated in DMEM/albumin alone or with 10 μg/ml apoA-I for a further 24 h. Cells were washed with PBS, fixed, and stained with filipin to measure intralysosomal unesterified cholesterol. Results are representative of three experiments with similar results. Bar = 20 μm.

Fig. 8. ABCA1-EGFP expression in NPC1 cells increases cholesterol available for esterification in the absence of apoA-I. Wild type (WT) fibroblasts or NPC1 fibroblasts with (+ ABCA1, open bars) or without Ad-ABCA1-EGFP infection were loaded with LDL and incubated in the presence of 1 mg/ml albumin alone or plus 10 μg/ml apoA-I (+ apoA-I) for 24 h. The cells were then washed and incubated with [14C]oleate for 1 h, and the cellular cholesteryl [14C]oleate formed as a measure of cholesterol available for esterification was determined as described in the Materials and methods. Values are the mean ± S.D. of triplicate determinations and are representative of two experiments with similar results. *, significantly lower than wild type fibroblasts incubated with albumin alone, p < 0.01. #, significantly higher than NPC1 fibroblasts uninfected with AdABCA1-EGFP and incubated with albumin alone, p < 0.01.

Fig. 9. ABCA1-EGFP expression in NPC1 cells suppresses HMG-CoAR expression in the absence of apoA-I. NPC1 fibroblasts were infected with Ad-EGFP or Ad-ABCA1-EGFP and loaded with LDL for 24 h. The cells were equilibrated in DMEM/albumin followed by incubation with DMEM/albumin alone or plus 10 μg/ml apoA-I for 24 h prior to determination of ABCA1 and HMG-CoA reductase by immunoblotting. Proteins were detected using 60 μg of total cell protein. Results are representative of three experiments with similar results.

4. Discussion

The results presented here provide several lines of evidence that overexpression of ABCA1 alone is able to correct the mobilization of cholesterol from late endosomes/lysosomes and the formation of HDL particles in NPC1– but not NPC2-deficient human fibroblasts. Cholesterol available for efflux to apoA-I following the loading of cells with LDL was increased to the same level as seen in normal cells by increasing expression of ABCA1 in NPC1 but not NPC2 cells. This effect was confirmed by the correction of the α-HDL particle formation by NPC1 cells but not NPC2 cells expressing ABCA1. Late endosome/lysosome cholesterol was markedly reduced in NPC1 but not NPC2 cells overexpressing ABCA1. Based on these results, a model can be proposed in which cholesterol delivered to late endosomal/lysosomal membranes by functional NPC2 can be mobilized either directly or indirectly from this compartment when ABCA1 expression is corrected, even in the absence of functional NPC1. In the absence of functional NPC2, however, cholesterol is not delivered to the lysosomal membrane and overexpressing ABCA1 is not able to induce cholesterol egress out of the late endosome/lysosome compartment.

These results are consistent with the demonstration that NPC2 binds cholesterol [34,6], and can deliver cholesterol to NPC1 [7] or to liposomal membranes [3,4,7]. Even in the absence of functional NPC1, if NPC2 is able to deliver cholesterol to the lysosomal membrane either via mutant NPC1 or the membrane itself, our model suggests this cholesterol then becomes available for removal following upregulation of ABCA1. Whether or not ABCA1 is removing this cholesterol from late endosomes/lysosomes directly through retroendocytosis, as some evidence suggests [13,25,27,28], or whether it indirectly causes a shift in lysosomal cholesterol to the plasma membrane following depletion of plasma membrane cholesterol by ABCA1 [29], or both, is not yet known. Previous studies have shown that expression of ABCA1–GFP by transfection or adenoviral gene delivery results in ABCA1 localization in the plasma membrane as well as intracellular compartments including late endosomes and lysosomes and the Golgi compartment [22–25]. Further experiments are required to determine whether this effect of ABCA1 in NPC1 cells is mediated at least in part by a direct interaction with ABCA1 and the lysosomal membrane to mobilize cholesterol. In the experiments presented here, apoA-I was not required to see depletion of late endosome/lysosome cholesterol in NPC1 cells following overexpression of ABCA1 (Fig. 7), suggesting the mobilization of cholesterol by ABCA1 at late endosomes/lysosomes and/or the plasma membrane does not require the presence or co-internalization of apoA-I.

These results are in interesting contrast to findings recently reported showing the mobilization of lysosomal cholesterol from both NPC1- and NPC2-deficient cells using cyclodextrins. In vivo studies in NPC1- [30] and NPC2-deficient mice [31] found treatment with 2-hydroxypropyl-β-cyclodextrin (HPCD) prolonged lifespan, corrected cholesterol regulation [30] and reduced intraneuronal cholesterol content [31] in these animals. Cell culture studies also showed reduced lysosomal cholesterol in both NPC1- and NPC2-deficient fibroblasts treated with either HPCD [32,33] or methyl-β-cyclodextrin [33], as well as correction of cholesterol esterification by ACAT1 in both cell types. Cyclodextrins are thought to be acting by either direct mobilization of cholesterol within endocytic organelles [33], or by inducing calcium-dependent exocytosis from lysosomes [34], bypassing both NPC2 and NPC1 defects. In contrast, our findings suggest overexpressed ABCA1 can bypass a defect of NPC1 to mobilize lysosomal membrane cholesterol, but does not mobilize cholesterol directly from the lysosomal compartment in the absence of NPC2 function.

Our results showing impaired ABCA1 expression in NPC2 cells, as also recently reported [35], underscore further the importance of lysosomally-derived cholesterol in the regulation of ABCA1. In addition to our original observation of this in NPC disease [11], we have found a similar defect in the lysosomal acid lipase deficiency cholesteryl ester storage disease [36], where the slowed hydrolysis of cholesteryl esters in lysosomes reduces the rate of flux of cholesterol from this compartment. In addition to regulating ABCA1 expression, the lack of major increase in HDL formation despite overexpressing ABCA1 in NPC2 cells (Fig. 6) suggests lysosomally-derived cholesterol forms a very large fraction of the cholesterol substrate used by ABCA1 for HDL formation. As seen for the cyclodextrins, and in the absence of apoA-I, cholesterol mobilized by ABCA1 from the late endosome/lysosome compartment is not only destined for cholesterol efflux but can also join the regulatory pool of cholesterol regulating cholesterol synthesis and esterification (Figs. 8 and 9). It is interesting to note than in 2003 Vaughan and Oram reported a similar increase of cholesterol esterification in baby hamster kidney cells expressing ABCA1, in the absence of any lysosomal cholesterol trafficking defect [37].

In summary, the current findings indicate that correction of the ABCA1 expression defect in NPC disease cells can overcome mutations in NPC1 but not NPC2 to mobilize lysosomal cholesterol, by a mechanism apparently quite different from that mediated by cyclodextrins. Treatments such as LXR agonists that correct the defect in ABCA1 expression in NPC disease cells would therefore be expected to have potentially similar effects as cyclodextrins in the majority of NPC disease patients.

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