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Functional ABCG1 expression induces apoptosis in macrophages and other cell types

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ABSTRACT

The expression of the ATP-binding cassette transporter ABCG1 is greatly increased in macrophages by cholesterol loading via the activation of the nuclear receptor LXR. Several recent studies demonstrated that ABCG1 expression is associated with increased cholesterol efflux from macrophages to high-density lipoprotein, suggesting an atheroprotective role for this protein. Our present study uncovers an as yet not described cellular function of ABCG1. Here we demonstrate that elevated expression of human ABCG1 is associated with apoptotic cell death in macrophages and also in other cell types. We found that overexpression of the wild type protein results in phosphatidyl serine (PS) translocation, caspase 3 activation, and subsequent cell death, whereas neither the inactive mutant variant of ABCG1 ($ABCG1_{K124M}$) nor the ABCG2 multidrug transporter had such effect. Induction of ABCG1 expression by LXR activation in Thp1 cells and in human monocyte-derived macrophages was accompanied by a significant increase in the number of apoptotic cells. Thyroxin and benzamil, previously identified inhibitors of ABCG1 function, selectively prevented ABCG1-promoted apoptosis in transfected cells as well as in LXR-induced macrophages. Collectively, our results suggest a causative relationship between ABCG1 function and apoptotic cell death, and may offer new insights into the role of ABCG1 in atherogenesis.

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1. Introduction

Since the concept of apoptosis was introduced in the early 70s by Kerr et al. [1], considerable knowledge on the physiological relevance of this phenomenon has accumulated. In a number of physiological processes the proper balance between cell proliferation and controlled cell death regulates organ development. The progression of atherosclerotic lesions is also determined by the balance of a series of pro- and antiatherogenic events. In recent years, apoptosis has been implicated as one of the key factors influencing the cellularity, stability, and thrombogenicity of the atherosclerotic lesions (for reviews see [2–4]).

Apoptosis in the atherosclerotic plaque can be either beneficial or harmful, depending on the cell type, the stage of the lesion, and the context. Apoptosis of endothelial cells and smooth muscle cells favors plaque instability [3–7], whereas macrophage apoptosis seems to be a double edged sword. In the early lesions, decrease in the number of macrophages is antiatherogenic as macrophage apoptosis results in a lower level of metalloproteinase activity and reduction in collagen breakdown, thus, increases plaque stability [2,3,8,9]. In contrast, in advanced atherosclerotic lesions, where the clearance of apoptotic bodies by intimal phagocytes becomes limiting [2,10,11], macrophage apoptosis is believed to promote atherogenesis. Loss of macrophages leads to further reduction in phagocytic activity, and with a positive feed-back mechanism results in the accumulation of non-scavenged apoptotic bodies, which are potential sources of inflammatory cytokines and tissue factors [12,13]. Thus this process leads to necrotic core formation, complement and thrombin activation. In addition, increased activity of metalloproteinases secreted by residual surviving macrophages reduces the stability of the atherosclerotic lesion [2,8,9]. Inflammation, thrombogenicity and augmented vulnerability can finally lead to plaque rupture. The molecular machinery regulating macrophage apoptosis under these circumstances, however, is not well understood.

Here we present evidence for an unexpected link between macrophage apoptosis and the activity of an ATP-binding cassette (ABC) transporter protein, ABCG1, highly expressed in cholesterol-loaded macrophages. The ABCG1 membrane protein belongs to the G subfamily of ABC transporters, the members of which are termed ‘reverse order’ half transporters. Unlike canonical ABC transporters, ABCG proteins consist of only a single nucleotide binding domain and a single transmembrane domain, which is localized C-terminally to the former one. An active ABC transporter requires the presence of at least two nucleotide binding domains and two transmembrane domains, therefore, the half transporters have to dimerize to form a functional unit. The ABCG1 transporter typically forms homodimers

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[14], but heterodimerization with ABCG4, the most closely related member of the ABCG subfamily, has also been demonstrated in our previous study [15].

In contrast to full length ABC transporters, which normally reside in the plasma membrane, several ABC half transporters localize to the membranes of intracellular organelles. Not only regarding dimerization, but also in terms of subcellular localization, ABCG1 seems to be a Janus-faced protein because both cell surface and intracellular expression of this transporter has been demonstrated [14,16,17]. Wang et al. proposed that a translocation of ABCG1 from the internal membrane to the cell surface occurs in response to LXR activation [16], although this notion was questioned by others [18]. Like most ABC transporters, ABCG1 possesses ATPase activity, which can be stimulated by putatively transported substrates [15], although direct transport of any substrate has not been demonstrated as yet. Also, the physiological substrates of ABCG1 remain to be identified.

Despite the growing number of studies on ABCG1, its physiological function is still elusive. Based on its homology with the Drosophila *White* gene product, which regulates the tryptophan and guanine uptake in fruit flies, the first studies on the mammalian ABCG1 predicted its contribution to the cellular transport of tryptophan, which is a precursor for serotonin, and suggested that the mutations in the human *ABCG1* gene may be associated with neurological disorders [19,20]. Subsequent studies did not provide further support for this hypothesis [21]. Recently, the most commonly accepted concept on the ABCG1 function is that this protein plays a role in cholesterol efflux in macrophages.

The involvement of ABCG1 in lipid homeostasis has been implicated by several studies demonstrating that the expression of ABCG1 in macrophages was induced by cholesterol load [22–24]. The possible connection of ABCG1 with lipid metabolism was further supported by the finding that its expression is regulated by LXR/RXR and PPARγ [25–27], the nuclear receptor systems that typically regulate genes controlling lipid metabolism. Direct cholesterol efflux from ABCG1-overexpressing cells to HDL and other lipoprotein particles has also been demonstrated by several studies [16,17,28–31]. Abolition of ABCG1 expression either by RNAi knockdown or by generation of knockout animals resulted in reduced cholesterol efflux to HDL [16,29,31,32]. Surprisingly, ABCG1 was unable to induce cellular cholesterol efflux to lipid-free apoA-I, the major apolipoprotein of HDL, to which ABCA1, another member of the ABC protein family mediates cellular efflux of cholesterol [33–35]. Based on this, ABCA1 and ABCG1 have been proposed to co-operate in the cellular removal of cholesterol. According to this model, ABCA1 facilitates loading of lipid-poor apoA-I with cholesterol, thus forming "nascent" HDL, which is further lipidated by the ABCG1-dependent cholesterol efflux [30,36–38].

The present work provides new insight into the possible function of the ABCG1 transporter. Here we demonstrate a link between the activity of ABCG1 and apoptotic cell death in macrophages and other cell types. These observations may extend our understanding of the formation and progression of atherosclerotic lesions.

2. Materials and methods

2.1. Expression of ABCG1 in Sf9 insect and mammalian cells

For infection of Sf9 (*Spodoptera frugiperda*) cells, the cDNAs of human ABCG1 and its mutant variant ($ABCG1_{K124M}$) were cloned into recombinant baculovirus transfer vectors as described previously [15]. For transfection of mammalian cells, the entire open reading frame of ABCG1 variants were cloned into pEGFP-N1 vectors (Becton Dickinson Clontech) leaving a stop codon and a frame shift between ABCG1 and eGFP sequences. The insect cell culturing and infection were carried out as detailed in [39]. HEK293 and MDCK cells were maintained in D-MEM containing 10% FCS, whereas HepG2 cells were cultured in F12 and D-MEM (1:1) +10% FCS. For transfection, the cells were seeded onto eight-well Nunc Lab-Tek II Chambered Coverglass (Nalge Nunc) at $1–3\times10^4$ per well cell density, and grown for 24 h. The cells were transfected with the DNA constructs by using the FuGENE 6 (Roche) reagent according to the manufacturer's instruction. The medium was changed 24 h after transfection, immunofluorescence and apoptosis studies were performed 48 h post-transfection.

2.2. Isolation of monocytes and culturing macrophages

Thp-1 cells were maintained in RPMI+10% FCS medium, and pretreated with 2 nM PMA 24 h before the induction of ABCG1 expression. Human monocytes were obtained from peripheral blood of healthy subjects by Ficoll separation followed by CD14 immunoisolation. Positive selection method was applied using CD14 MicroBeads and MiniMACS Separator (Miltenyi Biotec). The monocyte-derived macrophages were cultured in D-MEM containing 10% FCS for 5 days. The ABCG1 expression was induced in both PMA-pretreated Thp-1 cells and monocyte-derived macrophages by the addition of a synthetic LXR agonist, T0901317 (1 μM, Alexis Biochemicals) 8 or 24 h prior to quantitative RT-PCR and cell biology studies, respectively.

2.3. RNA isolation and real time RT-PCR

To obtain total RNA, 1×10^6 cells were dissolved in 1 ml Trizol (Invitrogen), and RNA was extracted according to the manufacturer's instructions. The purity of the RNA preparation was checked by measuring the absorbance ratio at 260/280 nm. Samples containing 1 μg of RNA were used for reverse transcription, which was carried out at 42 °C for 2 h and 72 °C for 5 min using Superscript II reverse transcriptase and Random hexamers (both from Invitrogen) according to the recommendation of the manufacturer. cDNA obtained was used for real-time quantitative PCR (ABI PRISM 7900, Applied Biosystems), 40 cycles of 95 °C for 10 s and 60 °C for 1 min. The sequence of the primers and probes are: ABCG1 (208+) TCCTCTTCAAGAGGACCTTCCT (284−) CCCAATGTGCGAGGTGAT (1233+) FAM-CATCATGAGGGACTCGGTCCTGACAC, cyclophilin: (52+) ACGGCGAGCCCTTGG (117−) TTTCTGCTGTCTTTGGGACCT (69+) FAM-CGCGTCTCCTTTGAGCTGTTTGCA. The comparative Ct method was used to quantify transcript levels and to normalize for cyclophilin expression.

2.4. Generation of monoclonal antibodies and Western blot analysis

Mice previously immunized with a GST-fused N-terminal domain of ABCG1, and producing ABCG1-specific sera as demonstrated in [15], were sacrificed and hybridoma cells were generated at the EMBL Antibody Core Facility (Monterotondo, Italy). The ELISA-positive hybridoma cells were cloned by limiting dilution and their supernatants were tested by Western blot analysis using ABCG1-containing Sf9 membrane preparations. Immunoblotting was performed as previously described [39]. The clones producing high level of antibody were expanded and their supernatants were enriched in the antibodies by step-wise serum deprivation. The supernatants were further concentrated by centrifugal ultrafiltering and the monoclonal antibodies were isotyped. Their selectivity was tested by immunoblotting using Sf9 membranes containing ABCG1, ABCG4, ABCG2, ABCG5, or ABCG5. Subsequent Western blot analyses and immunofluorescence studies were performed by using 6G1/7, a highly sensitive and specific, IgG1 isotype anti-ABCG1 monoclonal antibody.

2.5. Immunofluorescence staining

For immunofluorescence studies, the cells were gently washed with Dulbecco's modified PBS (DPBS), and fixed with 4% paraformaldehyde in DPBS for 5 min at room temperature. After 5 washes with DPBS, the samples were further fixed and permeabilized in pre-chilled methanol for 5 min at −20 °C. The cells were then blocked for 1 h at

room temperature in DPBS containing 2 mg/ml bovine serum albumin, 1% fish gelatin, 0.1% Triton-X 100, and 5% goat serum (blocking buffer). The samples were then incubated for 1 h at room temperature with 6G1/7, anti-ABCG1 antibody, diluted 100x in blocking buffer. After washing with DPBS, the cells were incubated for 1 h at room temperature with Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L) (Molecular Probes), diluted 250× in blocking buffer. For isotype control, mouse IgG1 (Sigma) and Alexa Fluor 488-conjugated goat anti-mouse IgG were used. The green fluorescence of stained samples was studied by an Olympus FV500-IX confocal laser scanning microscope using a UPLAPO 40× (0.85) dry or a PLAPO 60× (1.4) oil immersion objective (Olympus) at 488 nm excitation.

2.6. Cell death and apoptosis studies

For assessment of Sf9 cell death, the insect cells were infected with recombinant baculoviruses containing the sequences of the human ABCG1, $ABCG1_{KM}$, ABCG2, or β-galactosidase under identical circumstances (10^8 PFU virus per 10^7 cells) [15,39]. The fraction of living cells was determined 24, 36, 48, 72 and 96 h after infection by using Trypan-blue (Sigma) counterstaining.

To study apoptosis in mammalian cells, the cells were incubated with Alexa Fluor 488-conjugated Annexin V (Molecular Probes) in 1:20 dilution in Annexin binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$, pH: 7.4) containing 2 μM propidium iodide for 3 min. When indicated, the cells were preincubated with 100 μM thyroxin or 50 μM benzamil 24 h before apoptosis studies. In control experiments, apoptosis was induced by the addition of 2 nM staurosporine 5 h prior to studies. For combined PS translocation and caspase 3 studies, the cells were first incubated with a fluorogenic caspase 3 substrate, 10 μM $PhiPhiLuxG_2D_2$ (Calbiochem)+10% FCS (final conc.: 9 μM) for 20 min, washed twice and labeled with Alexa Fluor 488-conjugated Annexin V as described above. For combined PS translocation and immunofluorescence studies, the cells were first labeled with Alexa Fluor 488-conjugated Annexin V, washed, fixed, and immunostained as described above using Alexa Fluor 647-conjugated secondary antibody. The stained samples were studied with the confocal microscope specified above. The green, red, and deep red fluorescence were acquired between 505–525, 560–600 nm and above 650 nm, using 488, 543, and 633 nm excitations, respectively.

For quantitative evaluation of Annexin V binding, the labeled cells were counted in the fluorescence image, whereas the total cell number was determined in the corresponding DIC image. The fraction of Annexin V-labeled cells (% of total) was determined in 6–8 fields of view in several independent experiments ($n>3$). For statistical analyses, unpaired, 2-tailed, Student's t-test was used. Results are expressed as mean±s.e.m.

3. Results

In our previous work, we have biochemically characterized the ABCG1 transporter by using a heterologous baculovirus Sf9 insect cell system. In this transient expression system, transfection of the insect cells with baculovirus ultimately results in cell death. However, we have observed that the fraction of living cells in cultures expressing the wild type ABCG1 (wtABCG1) was significantly lower than in control cultures transduced with either β-galactosidase (β-gal), ABCG2, or the inactive, catalytic site mutant variant of ABCG1 ($ABCG1_{KM}$), even though the infection procedure were carried out under identical circumstances (10^8 PFU virus per 10^7 Sf9 cells) (Fig. 1). The proteins were expressed at similar high levels 72 h after transduction as observed by Coomassie staining or by Western blotting for ABCG1 and $ABCG1_{KM}$ (data not shown). The half life times determined from the kinetic analysis shown in Fig. 1, were 40.5± 1.2 h for cells transduced with the wild type ABCG1, and around 60 h for cells expressing $ABCG1_{KM}$, ABCG2, or β-gal. Accordingly, cells expressing wtABCG1 exhibited low accumulation of fluorescent viability markers, MitoTracker and calcein, as compared to their accumulation in cells expressing β-gal, ABCG2, or $ABCG1_{KM}$ (data not shown).

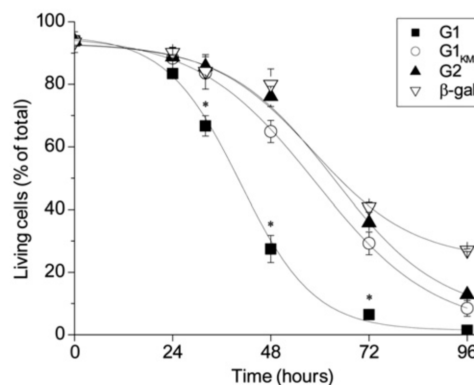

Fig. 1. Accelerated cell death caused by functional expression of ABCG1 in Sf9 cells. The wild type ABCG1 (filled square), its inactive mutant variant $ABCG1_{KM}$ (open circle), ABCG2 (filled up triangle), and β-galactosidase (open down triangle) were expressed in Sf9 heterologous expression system. The fraction of living cells was determined by Trypan-blue counterstaining at different time points after infection carried out under identical conditions (10^8 PFU virus per 10^7 Sf9 cells). Cells expressing the functional ABCG1 exhibited reduced viability as compared to cells expressing $ABCG1_{KM}$, ABCG2, or β-gal. Results are presented as mean±s.e.m. ($n=4$). Asterisks indicate significant difference between the viabilities of cells expressing wtABCG1 or $ABCG1_{KM}$ ($p<0.05$).

Previously, we demonstrated that the membrane cholesterol content substantially influences the activity of ABCG2 multidrug transporter [40]. Either cholesterol depletion or expression in cholesterol-poor Sf9 cell membrane results in reduced activity of ABCG2. Such a cholesterol-dependence has not been demonstrated for the activity of ABCG1. The significant difference seen between the viability of Sf9 cells expressing wtABCG1 or $ABCG1_{KM}$ indicates that ABCG1 remains active in the cholesterol-poor Sf9 membrane.

To explore whether the ABCG1-induced cell death can also be seen in mammalian cells, we expressed ABCG1 and its inactive variant ($ABCG1_{KM}$) in various cell lines (HEK-293, HepG2, COS-7, and MDCK cells). Mammalian cells transiently transfected with ABCG1 or $ABCG1_{KM}$ were immunostained with our monoclonal antibody specific for ABCG1 (6G1/7), and the morphology of the transfected cells was studied by confocal microscopy. As documented in Fig. 2, expression of wtABCG1 induced changes in cell shape with characteristic 'rounding up' and detachment, whereas cells expressing $ABCG1_{KM}$ exhibited normal cell morphology. In cultures transfected with wtABCG1, a substantial amount of cell debris, stained positively with the anti-ABCG1 monoclonal antibody, was also observed.

For subsequent functional studies we attempted to generate stable cell lines expressing the variants of ABCG1. Since the vectors used for transient transfection contained *neo*-resistance, the HEK-293 cells transfected with ABCG1 or $ABCG1_{KM}$ were subsequently selected with G418 for 10–14 days. Several attempts of generating a stable cell line expressing the wild type ABCG1 have failed, while we were able to obtain cells stably expressing the $ABCG1_{KM}$ mutant regularly. Furthermore, MDCK cells were transduced by retroviruses containing the ABCG1 and $ABCG1_{KM}$ sequences. The expression was frequently checked by Western blot analysis. After a short transient expression the cells lost the wild type protein, whereas cells transduced with the $ABCG1_{KM}$ variant stably expressed the protein (data not shown). These results are consistent with the observations of accelerated cell degradation seen in the ABCG1-transfected Sf9 cells, and implicated that the expression of the wtABCG1 also causes cell death in mammalian cells.

Since the morphological alterations observed in the ABCG1-expressing mammalian cells were indicative of apoptosis, next we examined several apoptotic markers in HEK-293 and HepG2 cells

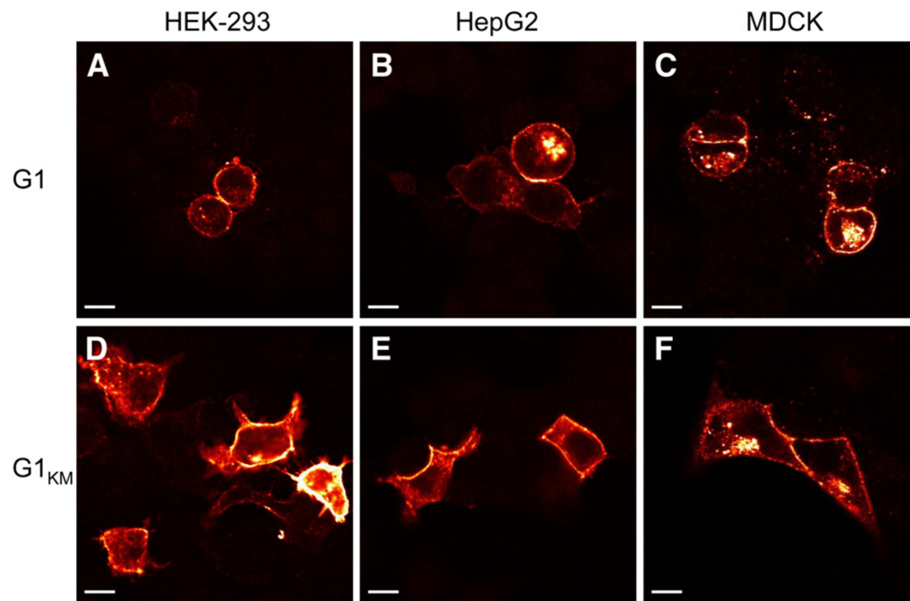

Fig. 2. Morphology changes associated with the functional expression of ABCG1 in various mammalian cells. HEK-293 (A, D), HepG2 (B, E) and MDCK (C, F) cells were transfected with the wild type ABCG1 (A–C) and the inactive mutant $ABCG1_{KM}$ (D–F). The cultures were immunostained with 6G1/7, an ABCG1-specific monoclonal antibody 48 h after transfection. Immunofluorescence was studied by confocal microscopy. Cells expressing the wild type ABCG1 protein were loosely attached and exhibited a 'rounded up' morphology, which is typical for apoptotic cells, whereas cells expressing $ABCG1_{KM}$ exhibited normal cell morphology. The white bars indicate 10 μm.

transiently transfected with wtABCG1 and $ABCG1_{KM}$. Annexin V binding was used to monitor the early apoptotic event of phosphatidyl serine (PS) translocation, while the activity of caspase 3, one of the key enzymes of apoptosis, was visualized by using the cell-permeable, fluorogenic caspase 3 substrate, $PhiPhiLuxG_2D_2$.

We found that the cultures transfected with wtABCG1 contained a large number of cells exhibiting Annexin V binding and elevated caspase 3 activity (Fig. 3A–C). The two apoptotic markers showed mostly overlapping patterns. In contrast, hardly any labeling for PS translocation and caspase 3 activity was seen in cultures transfected with $ABCG1_{KM}$ (Fig. 3D–G). The protein expression was verified by Western blotting in both cases (data not shown). It is important to note that despite caspase 3 and Annexin V positivity observed in the wtABCG1-expressing cells, membrane blebbing, another characteristic feature of apoptotic cells, or the condensation of the nuclei, as visualized by fluorescent nuclear staining, was not typically observed in these cells.

To investigate whether PS translocation is functionally associated with ABCG1 expression, Annexin V binding and ABCG1 expression were simultaneously investigated in HEK-293 and HepG2 cells transfected with wtABCG1 or $ABCG1_{KM}$. Close correlation between Annexin V binding and protein expression was observed in cultures transfected with the wild type protein (Fig. 4A–C). In contrast, no labeling with Annexin V was seen in cultures transfected with

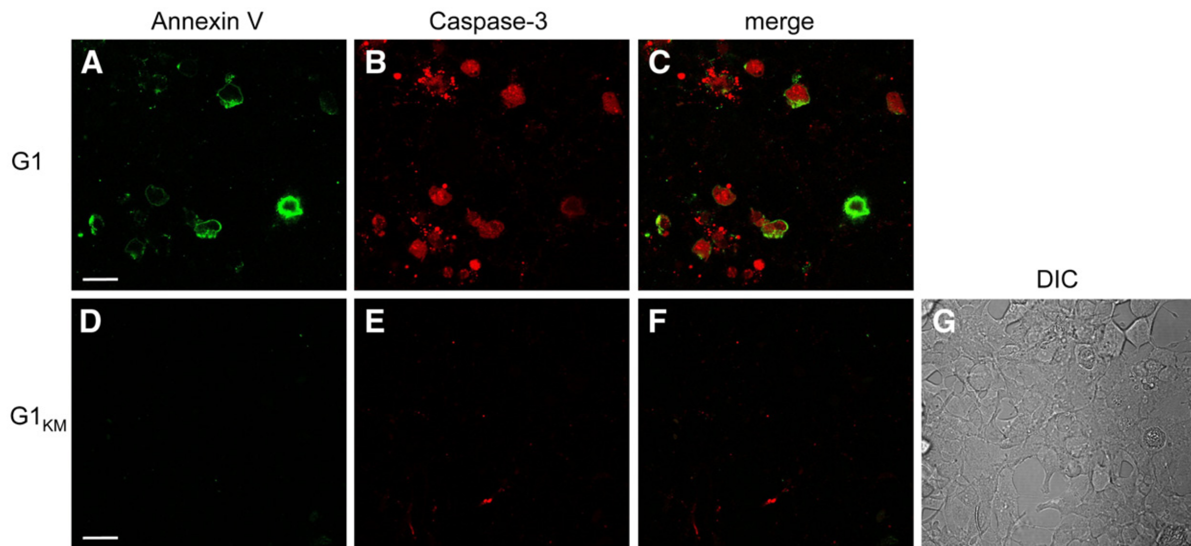

Fig. 3. Apoptotic markers in cell cultures transfected with ABCG1 or $ABCG_{KM}$. Phosphatidyl serine (PS) translocation and caspase 3 activity were assessed in HEK-293 and HepG2 cell transiently transfected with the wild type ABCG1 (A–C) or its inactive variant, $ABCG1_{KM}$ (D–G) 48 h after transfection. Representative experiments performed with HEK-293 cells are shown. PS exposure was visualized by the cell surface labeling of Annexin V conjugated with a green fluorophore (A, D), whereas the activity of caspase 3 was assessed by $PhiPhiLuxG_2D_2$, a cell-permeable caspase 3 substrate, which produces a red fluorescent product upon cleavage (B, E). Merged images are shown on the right (C, F). Substantial portion of cells were positively labeled for both PS translocation and caspase 3 activation in cultures transfected with the wild type protein, whereas hardly any staining was seen in $ABCG1_{KM}$-transfected cultures. The DIC image is to show the presence of the cells in the negatively stained sample. The white bars indicate 30 μm.

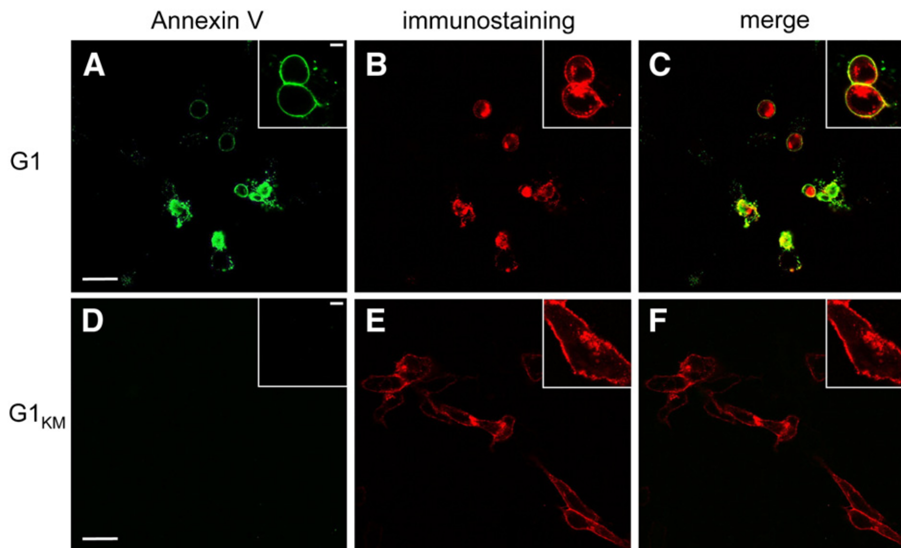

Fig. 4. Simultaneous assessment of phosphatidyl serine translocation and ABCG1 expression in cells cultures transfected with ABCG1 or $ABCG_{KM}$. HEK-293 and HepG2 cells transfected with ABCG1 (A–C) or $ABCG1_{KM}$ (D–F) were labeled with Alexa Fluor 488-conjugated Annexin V 48 h after transfection. The samples were subsequently fixed and immunostained with the ABCG1-specific monoclonal antibody, 6G1/7. Results obtained with HepG2 cells are shown. Annexin V binding (A, D) is depicted on the left, middle panels show the immunostaining (B, E), whereas merged images are shown on the right (C, F). In the ABCG1-transfected cultures, Annexin V binding was observed only in cells expressing ABCG1. In contrast, no labeling with Annexin V was seen in cultures transfected with $ABCG1_{KM}$, although the protein was expressed and localized normally. Similar results were obtained with HEK-293 cells. The white bars indicate 30 µm. Insets show a representative cell from the same field at higher magnification; bars: 5 µm.

$ABCG1_{KM}$ (Fig. 4D–F). The latter experiment also demonstrates that the mutant protein was expressed both on the cell surface and within intracellular compartments, similar to the wild type protein. It is important to note that apoptotic signs were only observed in cells expressing the ABCG1 protein (see Discussion).

The observation that the expression of the wild type ABCG1 resulted in morphological and functional alterations typical for apoptosis, whereas the inactive, catalytic site mutant variant exhibited no sign of apoptosis, suggested that the apoptotic events are related to the activity of the transporter. For exploring this question, in the subsequent experiments we employed thyroxin and benzamil, which were identified as efficient inhibitors of the ABCG1 ATPase activity in previous studies [15]. The quantitative analysis of Annexin V binding in ABCG1-transfected HEK-293 and HepG2 cells shown in Fig. 5 verified that the cultures transfected with the wild type ABCG1 contain significantly more Annexin V positive cells as compared to cultures transfected with the empty vector. However, preincubation of the cells either with thyroxin or benzamil completely blocked the ABCG1-induced apoptosis. Annexin V binding in $ABCG1_{KM}$-transfected cells was not different from the vector control, and was not affected by thyroxin or benzamil pretreatment.

For a comparison of the fraction of Annexin V positive cells in HEK-293 and HepG2 cell cultures, the values ought to be corrected by the transfection efficacy, which was about 30% in HEK-293 cells and 15% in

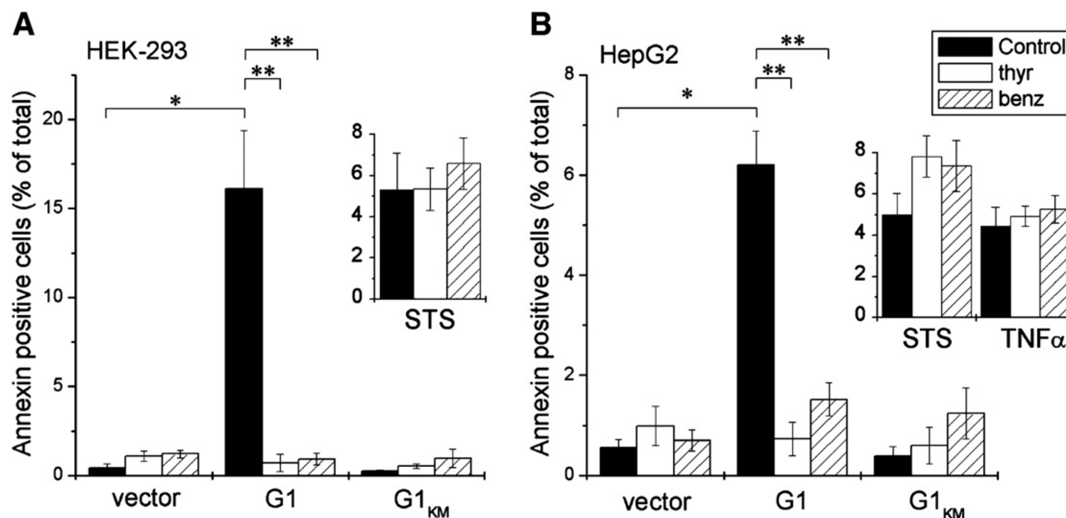

Fig. 5. Inhibition of phosphatidyl serine translocation in ABCG1-expressing cells with the inhibitors of ABCG1 function. The fraction of apoptotic cells was determined in HEK-293 (A) and HepG2 (B) cell cultures transfected with the empty vector, ABCG1 or $ABCG1_{KM}$. For quantitative analysis, the Annexin V-labeled cells were counted in the confocal fluorescence image, the total cell number was determined in the corresponding DIC image. Results are expressed as positively stained cells as percentage of total cell number. Between 1000 and 3000 cells were analyzed for each column in at least three independent experiments. Results are presented as mean ± s.e.m. The transfection efficiency for HEK-293 and HepG2 cells were 29.2 ± 3.5 and 12.1 ± 1.2, respectively. Significantly elevated number of apoptotic cells was seen in cultures transfected with wild type ABCG1 as compared to cultures transfected with the vector or $ABCG1_{KM}$ (black bars) (*, $p<0.001$ by Student's *t*-test). Preincubation with the previously identified inhibitors of ABCG1 function, thyroxin (thyr, white bars) and benzamil (benz, hatched bars), completely prevented PS translocation in the ABCG1-expressing cells (**, $p<0.001$ by Student's *t*-test). These compounds had no effect on Annexin V binding in cells transfected with the empty vector or $ABCG1_{KM}$, neither in cultures, in which apoptosis was induced by staurosporine (STS) or TNFα (see insets).

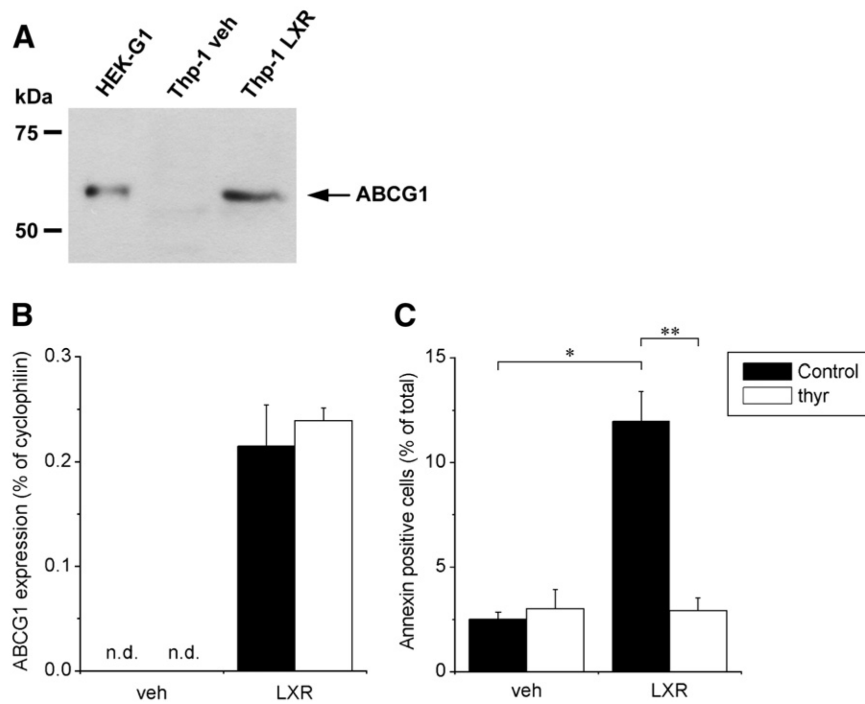

Fig. 6. Induction of ABCG1 expression in Thp-1 cells by LXR agonist; assessment of apoptosis in the LXR-induced Thp-1 cells. PMA-pretreated Thp-1 cells were subjected to 1 μM T0901317, an LXR agonist, and expression of endogenous ABCG1 expression was detected by Western blotting (A) and quantitative PCR (B). Western blot analysis was performed with total cell lysates (50 μg protein) using 6G1/7, ABCG1-specific antibody. As a positive control, HEK-293 cells transfected with ABCG1 (5 μg protein) was used. Considerable elevation in the ABCG1 expression was observed at both mRNA and protein levels in response to the LXR agonist. Quantitative Annexin V binding experiments were performed with PMA-pretreated Thp-1 cells (C) as described in detail in the legend of Fig. 5. In parallel with the induction of ABCG1 expression, T0901317 significantly increased the number of apoptotic cells (black bars). This elevation was completely inhibited by the pretreatment with 100 μM thyroxin (thyr), a blocker of ABCG1 function (C, white bars), without any effect on the expression level of the transporter (B, white bar). Results are expressed as mean ± s.e.m. Asterisks indicate significant differences: *, when compared with vehicle control; **, when compared with cells not-pretreated with thyroxin by Student's *t*-test ($p<0.005$). n.d.: not detected.

HepG2 cells, respectively. Thus, about 50% of the transfected cells exhibited Annexin V labeling at the examined time point, i.e., 48 h after transfection. In order to demonstrate the relative specificity of the effect of thyroxin and benzamil on ABCG1-induced apoptosis, in parallel experiments apoptosis was induced by staurosporine both in HEK-293 and HepG2 cells. As shown in Fig. 5, the fraction of Annexin positive cells in the staurosporine-treated cultures was not reduced by the ABCG1 inhibitors. Similarly, Annexin V binding in TNFα-induced HepG2 cells was not affected by the pretreatment with thyroxin or benzamil.

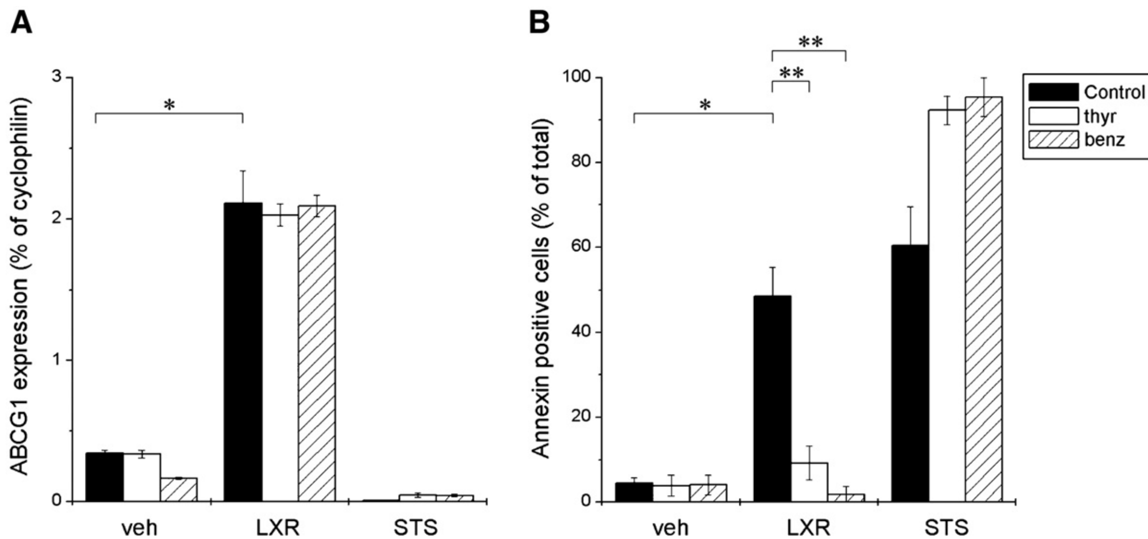

Fig. 7. Parallel induction of ABCG1 expression and apoptosis in human monocyte-derived macrophages and inhibition of apoptosis with the ABCG1 inhibitors. Human monocyte-derived macrophages were obtained from peripheral blood by CD14 immunoisolation. After 5 days of culturing, the cells were incubated with 1 μM T0901317, and the expression level of ABCG1 was measured by quantitative PCR (A). In parallel the fraction of apoptotic cells was determined by Annexin V binding (B). Both ABCG1 expression level and number of apoptotic cells were significantly increased in response to LXR activation (black bars). Preincubation with 100 μM thyroxin (thyr, white bars) or 50 μM benzamil (benz, hatched bars), prevented PS translocation in the LXR-ligand-induced cells, without any effect on ABCG1 expression. To exclude a non-specific effect of thyroxin and benzamil on the Annexin V binding, in control experiments apoptosis was induced in the monocyte-derived macrophages by staurosporine (STS) pretreatment. These compounds did not block Annexin V binding in the STS-induced apoptotic cells. ABCG1 expression was not induced by staurosporine (A). Results are expressed as mean ± s.e.m ($n=4$). Asterisks indicate significant differences: *, when compared with vehicle control; **, when compared with non-treated cells by Student's *t*-test ($p<0.001$).

In order to explore whether the ABCG1-induced apoptosis is solely a consequence of forced expression of the transporter, or the phenomenon also occurs in cell types that endogenously express ABCG1, we investigated Annexin V binding in PMA-pretreated human Thp-1 cells (a macrophage model system), and in isolated human monocyte-derived macrophages. The endogenous ABCG1 expression in these cell types was induced by T0901317, a known activator of LXR, the nuclear receptor that regulates *ABCG1* gene [26,27].

As shown in Fig. 6, in Thp-1 cells the expression level of ABCG1 was studied by both quantitative PCR and Western blotting and, in parallel, the fraction of apoptotic cells was determined by Annexin V binding. As expected, a marked induction in the ABCG1 expression was seen in response to LXR activation (Fig. 6A, B). In parallel, hardly any Annexin V positivity was seen in the control cells, which were treated with the vehicle only, whereas substantial Annexin V labeling was observed in the LXR-induced cultures (Fig. 6C). Similar to that seen in the ABCG1-transfected cell lines, preincubation with thyroxin prevented Annexin V labeling in the LXR-ligand-induced Thp-1 cells.

In similar experiments, carried out with monocyte-derived human macrophages, a substantial induction of ABCG1 expression and a subsequent elevation of the fraction of apoptotic cells were seen in response to LXR agonist (Fig. 7). Preincubation with thyroxin and benzamil completely blocked Annexin V labeling in the LXR-ligand-induced macrophages without affecting the ABCG1 expression. These observations indicate that the PS translocation in the LXR-induced macrophages depends on the functional presence of ABCG1 protein. To exclude a non-specific effect of thyroxin and benzamil on Annexin V binding, apoptosis was also induced by staurosporine in these cells. As shown in Fig. 7, the expression of ABCG1 was not elevated by staurosporine, and the marked Annexin V binding observed in the staurosporine-induced cells was not reduced by thyroxin or benzamil pretreatment (rather a small increase was detected).

4. Discussion

In order to explore the functional role of the human ABCG1 membrane transporter protein, in the present experiments we explored the effects of ABCG1 expression in numerous cell types, including human macrophages involved in atherosclerosis development. Previous studies suggested that overexpression of ABCG1 causes an increase in cholesterol efflux from cells to HDL and other lipid acceptors [16,17,28–31]. In addition to such a possible role, our present experiments suggest another cellular function for ABCG1, and may offer new insights into the function of this transporter protein.

Here we demonstrate that ABCG1 functionally contributes to apoptotic events in various cell types, the function of the over-expressed protein is closely connected to cell death, activation of caspase 3, and PS translocation. We show that physiological ABCG1 expression, induced by the addition of a synthetic LXR agonist in monocyte-derived macrophages or PMA-treated Thp-1 cells, also results in a substantial increase in phosphatidyl serine surface exposure. In all cases the function of ABCG1 is required to evoke these apoptotic signs.

Several previous studies demonstrated an induction of ABCG1 expression in macrophages in response to cholesterol loading [22,23,41], whereas other studies showed PS translocation in macrophages induced by cholesterol [42,43]. However, the causative connection between ABCG1 and apoptotic events was documented in our present study by the effects of ABCG1 inhibitors on PS exposure. In addition, the fact that the ABCG1-transfected cells exhibited PS surface exposure, whereas this was not observed in cells transfected with the inactive ABCG1 variant ($ABCG1_{KM}$), clearly indicates an association between the PS translocation and the transport activity of ABCG1.

A distant relative of ABCG1 in the ABC protein family, MDR3 (ABCB4) is known as a phosphatidyl choline (PC) floppase, translocating PC from the inner membrane leaflet to the outer leaflet. Based on this homology, we may hypothesize that ABCG1 acts as a PS translocator. However, PS surface exposure in the ABCG1-expressing cells is most likely not a consequence of direct floppase activity, but a component of a concerted apoptotic process, since caspase 3 activation as well as accelerated cell death were observed in cells expressing the wild type ABCG1. None of these apoptotic events was seen in cells expressing the inactive, catalytic site mutant variant of ABCG1 ($ABCG1_{KM}$), although the expression level and the subcellular localization of the mutant form was similar to those of the wild type protein. Therefore, our results imply a functional association between macrophage apoptosis and ABCG1 transport activity.

Numerous studies reported collateral sensitivity to certain compounds along with the overexpression of ABCB1 (MDR1/Pgp) or ABCC1 (MRP1). These drugs exemplified by gemcitabine, cytosine arabinoside, arsenic trioxide, and NSC73306 for ABCB1 [44–47], as well as verapamil, its derivative, $NMeOHI_2$, cytosine arabinoside, melphalan, apigenin, and buthionine sulphoximine for ABCC1 [48–51]. Although the mechanism behind this collateral sensitivity is still elusive, it has been suggested that at least in ABCC1-expressing cells the decreased glutathione level content and the subsequent oxidative stress account for the hypersensitivity to drugs causing further reduction in glutathione content. Since in our study no exogenous drug was added to the cells, some endogenous component(s) can be responsible for the ABCG1-promoted cell death.

There are several factors that are known to induce apoptosis in macrophages. These include ATP depletion, binding of death receptors ligands, growth factor withdrawal, high concentration of oxidized LDL, oxysterols, and intracellular accumulation of unesterified or "free" cholesterol [2,42,43,52]. In addition to their apoptotic effect, these agents are also known to induce ABCG1 expression in macrophages [22,23,27,41,53], which is in accordance with our present results.

Regarding the ABCG1-dependent apoptosis found in this study, several possibilities can be excluded from the list of the aforementioned pro-apoptotic factors. ATP depletion cannot account for the ABCG1-dependent apoptosis, since overexpression of a closely related ABC transporter, ABCG2, which possesses higher ATPase activity, does not result in PS translocation, caspase 3 activation, and subsequent cell death (see Fig. 1). The involvement of death receptors is also unlikely, since TNFα-induced apoptosis was not blocked by the inhibitors of ABCG1.

A possible explanation for the mechanism of ABCG1-dependent apoptosis could be that the transporter may act as an export pump, and mediate the excretion of certain cytotoxic compounds to the media, which cause apoptosis in the adjacent cells. Recently, it has been suggested that 7β-hydroxycholesterol or 7-ketocholesterol is expelled from cells by ABCG1 [54,55]. In order to explore this possibility, we examined whether the apoptotic effect of ABCG1 is restricted to the cell that expresses the transporter or a paracrin effect can be seen on the adjacent cells. As shown in Fig. 4B, PS translocation was observed solely in ABCG1-expressing cells, and no signs of apoptosis can be seen in the neighboring cells. In addition, we co-cultured GFP-expressing cells with ABCG1-transfected cells, but no apoptotic GFP-expressing cells adjacent to the ABCG1-expressing cells were seen (data not shown). Based on these experimental observations the paracrin model seems to be unlikely.

As mentioned above, intracellular accumulation of unesterified or free cholesterol is one of the apoptotic inducers in lesional macrophages. It has been demonstrated that free cholesterol-induced apoptosis is a multifactorial process, which involves ER stress caused by free cholesterol trafficking to ER, which, in turn, results in unfolded protein response (UPR), and activation of the C/EBP homologous protein (CHOP) [56–58]. Other cellular events that greatly influence free cholesterol-induced macrophage apoptosis include activation of c-Jun N-terminal kinase (JNK), biding of scavenger receptor A (SRA)

ligands, cellular calcium perturbation, and activation of group VIA phospholipase A_2 (iPLA$_2\beta$) [57–60]. The requirement of cholesterol trafficking to the ER is demonstrated by the inhibitory effect of selective blockers of cholesterol trafficking [56,61], and by the reduced macrophage apoptosis in advanced lesion in mice with a heterozygous mutation in the cholesterol-trafficking protein Niemann-Pick C1 [62]. Given that ABCG1 is likely involved in cholesterol transport and it exhibits both cell surface and intracellular expression [14,16,17,36,63], we can speculate that elevated expression of this transporter results in ER stress, which in turn, leads to apoptosis. This hypothesis presumes that the transport activity of ABCG1 surpasses the cholesterol esterification capacity of the cell, which results in accumulation of free cholesterol in the ER. However, this speculative model needs to be investigated and experimentally justified.

At present the most widely accepted concept for the physiological function of ABCG1 is that this transporter expels cholesterol from macrophages and other cell types to HDL. It is a plausible explanation for the ABCG1-induced apoptosis that the observed cell death is a consequence of the cholesterol efflux associated with the functional ABCG1, since neither the ABCG1$_{KM}$- nor the ABCG2-expressing cells exhibited this phenotype. Although it was previously suggested that ABCG2 stimulates cellular efflux of sterols including cholesterol [64], this hypothesis was based on the observation that sterols stimulate the ATPase activity of ABCG2 expressed in bacterial membrane lacking cholesterol. To our recent understanding ABCG2 does not expel cholesterol but its ATPase activity is allosterically stimulated by cholesterol [40]. Nevertheless, cholesterol depletion itself did not significantly increase the number of apoptotic cells in HEK-293 culture pretreatment with 4 mM cyclodextrin for 30 min, despite the fact that about 25% decrease in total cholesterol content was achieved with this treatment (data not shown).

The hypothesis that ABCG1 extrudes cellular cholesterol to HDL is based on numerous in vitro and in vivo studies demonstrating cellular cholesterol efflux in connection with ABCG1 expression [14,16,17,28–32,36,55]. However, the specificity of this transport process is questioned by the fact that similar ABCG1-mediated cholesterol efflux was seen to various lipid acceptors, such as LDL, PC vesicles, PC/ApoA-I discs, even BSA and cyclodextrin [14,16,31,36]. The observation that Ldr −/− mice transplanted with ABCG1 −/− bone marrow exhibited reduced cholesterol level in lipoproteins other than HDL [55], makes the specific role of HDL in ABCG-mediated cholesterol efflux even more tentative. Our recent finding, that ABCG1 promotes macrophage apoptosis, provides an alternative explanation for the non-specific cholesterol efflux in connection with ABCG1 expression. Our observation suggests that cholesterol efflux can be a consequence of cell death induced by ABCG1. This model is also consistent with the suggested protective role of ABCG1 in the atherogenesis [38,65].

Another concern that adds complexity to the issue of ABCG1-dependent apoptosis is that two recent studies suggested that ABCG1 diminishes the apoptotic effect of 7β-hydroxycholesterol or 7-ketocholesterol on macrophages [54,55]. This apparent contradiction can be resolved if we take into consideration that these studies demonstrated increased susceptibility to exogenously added cytotoxic compounds when ABCG1 was defective (either by using an inactive mutant form, or cells from ABCG1 knockout animals), whereas our study is based on endogenous transport activity of ABCG1. Since the externally added oxysterols at low concentrations can act as competitive inhibitors of the endogenous substrate(s), addition of these compounds can result in a decreased cell death in cells possessing active ABCG1. In accordance with this concept, only a slight and transient change in the dose–response relations was seen in connection with ABCG1 in the aforementioned studies [54,55]. An alternative explanation for the notion that ABCG1 can act both as an inducer and inhibitor of macrophage apoptosis is that the transporter may play different roles, depending on its subcellular localization. When the transporter is located in the plasma membrane, it can expel cytotoxic compounds from the cell, whereas it can stimulate apoptosis, when located in the internal membranes, e.g., can induce an ER stress.

Whether ABCG1-dependent apoptosis of macrophages has a physiological and clinical relevance, our findings should be evaluated in the context of studies in animal model systems of atherosclerosis. These studies demonstrated an inverse relationship between macrophage apoptosis and the early lesion area, and concluded that macrophage apoptosis is physiologically beneficial in the early atherosclerotic lesions [2,3,66–69]. In contrast, loss of macrophages becomes detrimental in the advanced lesions, where phagocytic activity becomes limiting [2,3,10,11]. With respect to the role of ABCG1 in atherogenesis, recent in vivo studies have led to contradicting results, demonstrating either an increase or a reduction in lesion size in mice transplanted with ABCG1-deficient bone marrow [70–75]. It has been hypothesized by L. K. Curtiss in an editorial [76] that the different degree of hyperlipidemia achieved in the recipient animals, and consequently the different progression rate of atherosclerosis can account for these conflicting results. Our recent finding, the phenomenon of ABCG1-promoted apoptosis, is in accordance with this hypothesis, and can reconcile the disparate results of ABCG1 transplantation studies. Since macrophage apoptosis can be both atherogenic and atheroprotective, ABCG1, which may be involved in the apoptotic processes, can also have a dual role in the pathogenesis of atherosclerosis, depending on the progression of atherosclerosis.

Although the above discussion focused on macrophage apoptosis, the ABCG1-promoted apoptosis described here might have a role in other cells types as well, in which this transporter is expressed in an inducible manner. These include vascular endothelial cells, astrocytes, microglia, and neuronal cells [28,63,77–81]. Given that apoptotic cell death has a crucial role in neuroprotection and normal vascular function, it is an intriguing possibility that ABCG1 expression and function contributes to apoptotic processes in these tissues.

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