

Overexpression of ABCG1 protein attenuates arteriosclerosis and endothelial dysfunction in atherosclerotic rabbits

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Abstract

The ABCG1 protein is centrally involved in reverse cholesterol transport from the vessel wall. Investigation of the effects of ABCG1 overexpression or knockdown *in vivo* has produced controversial results and strongly depended on the gene intervention model in which it was studied. Therefore, we investigated the effect of local overexpression of human ABCG1 in a novel model of vessel wall-directed adenoviral gene transfer in atherosclerotic rabbits. We conducted local, vascular-specific gene transfer by adenoviral delivery of human ABCG1 (Ad-ABCG1-GFP) in cholesterol-fed atherosclerotic rabbits *in vivo*.

Endothelial overexpression of ABCG1 markedly reduced atheroprotection (plaque size) and almost blunted vascular inflammation, as shown by markedly reduced macrophage and smooth muscle cell invasion into the vascular wall. Also endothelial function, as determined by vascular ultrasound *in vivo*, was improved in rabbits after gene transfer with Ad-ABCG1-GFP. Therefore, both earlier and later stages of atherosclerosis were improved in this model of somatic gene transfer into the vessel wall. In contrast to results in transgenic mice, overexpression of ABCG1 by somatic gene transfer to the atherosclerotic vessel wall results in a significant improvement of plaque morphology and composition, and of vascular function *in vivo*.

in this family of at least 51 different genes with different specificities. The structure of ABCG1 is that of a transmembrane protein, which consists of short extracellular loops, six transmembrane domains, and an intracellular ATP-binding cassette.

Mice that were transplanted with bone marrow from ABCA1 knockout mice are characterized by increased atherosclerotic lesion development,⁶ whereas mice that were transplanted with bone marrow from ABCA1 over-expressing mice showed less atherosclerosis.⁷ Consistently, ABCA1-deficient macrophages demonstrated reduced RCT *in vivo*.⁸ Increased ABCA1 activity and overexpression of ABCA1 protect against atherosclerosis.^{9,10}

ABCA1 primarily regulates phospholipid and/or cholesterol efflux. Whether one is more important than the other remains controversial. In contrast to ABCA1, ABCG1 facilitates cholesterol efflux to mature HDL, but not to lipid-poor apoA-I.^{11,12} A synergistic relationship between ABCA1 and ABCG1 in peripheral tissues has been proposed in which ABCA1 lipidates any lipid-poor/free apoA-I to generate nascent or pre-beta-HDL which could serve as substrates for ABCG1-mediated cholesterol export.

Macrophages of mice which were deficient in ABCG1 demonstrated lipid accumulation when fed a high-fat high-cholesterol diet,¹² and impaired cholesterol efflux to HDL *ex vivo*, together with significantly reduced RCT *in vivo*.⁸ In contrast to the more clear-cut findings with ABCA1, however, such a macrophage deficiency of ABCG1 was associated with decreased atherosclerosis in LDLr (-/-) as well as in Apo E (-/-) mouse models,^{13,14} when only ABCG1 and not ABCA1 was ablated. In contrast, double knockdown of both ABCA1 and ABCG1 in macrophages strongly reduced cholesterol efflux *ex vivo* and RCT *in vivo*.⁸ Consistently, but in contrast to the findings with single ABCG1 knockout, transplantation of ABCA1/ABCG1 double knockout bone marrow into atherosclerosis-prone mice resulted in substantially greater atherosclerosis than loss of function of either transporter alone.¹⁵ Crossing ABCG1-overexpressing mice with

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Introduction

Reverse cholesterol transport (RCT) is a physiological process by which cholesterol is transported by high density lipoproteins (HDL) from peripheral tissues to the liver for further processing and excretion.¹ Promotion of cholesterol efflux, the first step of RCT, could be an effective therapeutic strategy to reduce risk of atherosclerotic vascular disease.²

The superfamily of ATP-binding cassette (ABC) transporters consists of multiple multi-transmembrane proteins which transport various molecules across extra- and intracellular membranes. The subtypes ABCA1 and ABCG1 are expressed, among others, in endothelial cells and macrophages, and regulate cellular cholesterol and phospholipid homeostasis. These two subtypes belong to a small group within the transporter family which facilitates the cellular efflux of cholesterol. In endothelial cells, ABCG1 is the most prominent family member mediating cholesterol efflux to HDL whereas, in these cells, ABCA1 seems to be less important.³⁻⁵

ATP binding transporter proteins have been described as key mediators of cholesterol efflux out of hepatic tissue, macrophage, monocytes and endothelial cells.³⁻⁵ The subtypes ABCA1 and ABCG1 play a dominant role

LDLr (-/-) mice led to increased atherosclerosis in these animals.¹⁶ However, a recent study on whole-body overexpression of human ABCG1 in Apo E -/- mice did not show relevant changes of either plasma lipids or atherosclerosis.¹⁷

Obviously, these different findings are rather difficult to reconcile. They might partly be explained by the life-long knock-out or overexpression of the transgene in all these animal models, which might have obscured the true effects by counterregulation of other ABC family members.

Therefore, we investigated the effect of an overexpression of human ABCG1 on the progression of atherosclerosis and endothelial function after local gene transfer of this protein into adult carotid arteries, by using a novel model system.¹⁸

Material and Methods

Ad-ABCG1-GFP construction

A PCR cloning technique was used to generate an adenovirus coding for human ABCG1. For ABCG1, the forward primer *cgcggggtaccacatggccgtttctcgtg* and reverse primer *cgcggggcgccg cctctctcccggattttgt* were used, corresponding to the sequence published under accession n. NM004915.2. The complete sequence of the transcript was amplified with the respective primer pairs from a HUVEC cDNA library by PCR amplification. The PCR fragment of ABCG1 (KpnI/NotI) was cloned together with a PCR-amplified GFP fragment (NotI/XhoI) using the primers *cgcggggcgccgc-catggtgagcaaggcgagg* and *cgcgggctcgagtcactgtacagctctccatgc* into the plasmid pShuttle CMV, generating the plasmid pShuttle CMV ABCG1-eGFP encoding the complete human ABCG1 sequence in fusion with eGFP.

The plasmid pShuttle CMV ABCG1-GFP was linearized with PmeI (New England Biolabs, Beverly, MA, USA) and co-transfected with pAdEasy1 for recombination in electrocompetent *E. coli* BJ5183 (Stratagene, La Jolla, CA, USA). Positive colonies were checked for the recombinant adenoviral plasmid and further used for transfection into HEK 293 cells. After plaque isolation by GFP fluorescence, recombinant virus particles (Ad-ABCG1-GFP) were further amplified in HEK 293, purified, and stored at -80°C. A control adenovirus containing only the reporter gene GFP (Ad-GFP) was amplified and purified in the same manner. To avoid unspecific alterations caused either by the adenovirus infection itself or the reporter gene GFP, effects of Ad-ABCG1-GFP (co-expressing GFP) were compared to Ad-GFP-only infected respective controls.

Transgene expression in cultured human umbilical vascular endothelial cells

Commercially available low-passage number human endothelial cells (HUVEC) were seeded in 96-well plates and cultured to a confluence of 70%. After gene transfer, cells which overexpressed ABCG1-GFP showed a clear green fluorescence, as much as cells which overexpressed GFP only. Viral gene transfer had an efficiency of almost 100%, so that almost all cells harbored the respective transgenes.

Western blotting was performed according to standard techniques with the anti-human ABCG1 antibody NB400-132 from Novus Pharma, Littleton, CO, USA.

In order to determine cholesterol efflux from HUVECs, the cells were incubated in serum- and lipoprotein-free medium with 5 µg/mL cholesterol (synthetically generated, cell-culture tested, Sigma-Aldrich) for 24 h. Cells were thoroughly washed with PBS, and fresh colorant-free medium was added which contained no cholesterol but either 100 µg/mL HDL (Biomedical Technologies, Stoughton, MA, USA cat. n. BT-914), or no additive. Before starting efflux, intracellular cholesterol contents were measured to determine the baseline contents for the control dishes after harvesting cells. Spiked cholesterol was used as an additional internal standard. After adding media, samples were taken to measure initial cholesterol content. After the efflux period, cells and supernatant (media) were collected separately. Specific cholesterol was determined by HPLC-MSMS using an Inertsil ODS column on an

Agilent 1100 device at Currenta Analytics GmbH, Leverkusen, Germany. Specific cholesterol efflux was determined as the difference of cholesterol release to HDL-containing supernatant immediately after adding media and at the end of the experiment *versus* medium only on GFP-expressing cells within 16 h expressed as parts per billion (ppb). Percentages of sterol efflux were calculated by the ratio of sterol content in the medium to total cholesterol content in medium and cells.

Animals

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication n. 85-23, revised 1996). New Zealand White rabbits weighing 3.6±0.3 kg (Asam, Aretsried, Germany) were kept under constant temperature and humidity with standard feed according to animal health regulations. Specific permission for all animal studies was obtained from the local animal safety supervising authority, the Ethics Committee of the government of the State of Bavaria (study ns. 55.2-1-54-2531-43-05 and 55.2-1-54-2531-113-01 of the government of the State of Bavaria). For the induction of atherosclerosis, rabbits were fed with a high cholesterol (0.25%) diet for eight weeks. After four weeks of high cholesterol diet, vascular gene transfer was performed.

Vascular gene transfer to the carotid artery

Animals were subjected to general anesthe-

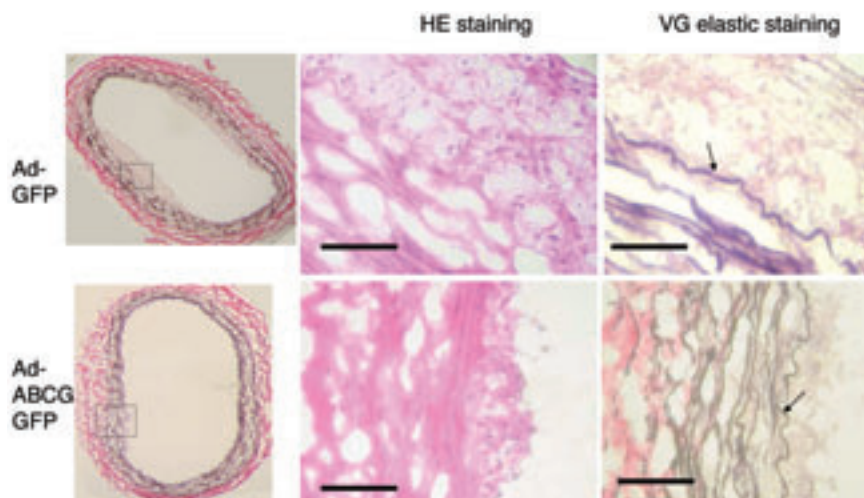


Figure 1. Representative histological sections of the left carotid artery: significant plaque formation in GFP-infected cholesterol-fed rabbits (Ad-GFP) as determined after staining with hematoxylin eosin and elastica van Gieson (VG) staining. Local Ad-ABCG1-GFP infection of the left carotid artery resulted in reduced plaque formation. The scale bar corresponds to a length of 50 µm. The different thicknesses of the intima surrounded by elastic tuna (arrow) in VG elastic staining are shown.

sia with propofol (propofol 2%, Fresenius) and fentanyl (Fentanyl-Janssen 0.5 mg, Janssen-Cilag, 0.01 mg/kg). They were intubated and artificially ventilated (AWS, Fa. Völker, Germany). For analgesia, all animals received buprenorphin (Temgesic®, Boehringer, 0.01 mg/kg) and 100 IU/kg heparin before surgery. A cervical midline incision was made and the left common artery was exposed. A segment of 4 cm was isolated with two small atraumatic clips (BIEMER vessel clips, FD 561 R). Approximately 0.2 mL virus solution (titer of 1×10^{10} pfu) was injected by a small needle (0.4×20) into the isolated segment. Incubation time was 20 min. Then the clips were removed and the blood circulation was restored. The cervical wound was sutured and the animals were allowed to recover. The rabbits received analgesia (Temgesic®, buprenorphin 0.01 mg/kg sc every 12 h) for 72 h post surgery.

Upon establishing the project, vessel wall sections were used to investigate the intactness of the endothelial coverage. No major damage to the endothelium occurred, no signs of endothelial denudation were seen 1-3 days after gene transfer nor at later time points (see endothelium outside plaques in Figure 1, left panel).

Assessment of endothelial dysfunction and wall thickness *in vivo*

Four weeks after gene transfer and eight weeks after the start of cholesterol feeding, rabbits were subjected to general anesthesia and ventilated as described above. Vascular ultrasound was performed in dorsal position with fixation of the ultrasound probe. Flow measurements were performed in color

Doppler mode and vascular wall thickness was measured by 2D ultrasound. Endothelial dysfunction was assessed by measuring flow-induced endothelial dependent vasoreactivity after a volume challenge with 80 mL NaCl IV injected via an ear vein. Vascular diameter was measured and the change of the diameter was expressed in percentage of the baseline diameter. Wall thickness was measured in mm. After measurements had been taken, rabbits were killed by an overdose of pentobarbital for post-mortem.

Histological assessment of atherosclerosis

The left and right carotid arteries, aorta and iliac arteries were macroscopically prepared for *en face* evaluation of plaque extension and stained with Sudan III. Arteries were opened longitudinally and pinned out flat on a blue wax surface with the endothelium facing up. Digital macroscopic images were taken and plaque extension was determined by computer-assisted image analysis (Scion image) calculated in percentage of the vessel area.

Additionally, a segment (length 5 mm) from the middle of the left and the right common carotid arteries was embedded in OCT compound (Leica, Germany) and frozen in liquid nitrogen.

First, freeze-cut sections of the left and right carotid arteries (40 μm) were viewed with UV light for GFP-specific fluorescence. Sections were viewed with a Zeiss Microscope (×10 magnification) and photographed.

Secondly, serial cryosections (6 μm) were cut from the same middle segments of the arteries. For histological assessment of atherosclerosis, hematoxylin and eosin (HE) and van Gieson (VG)-elastica staining were per-

formed. Digital images were taken and the intima and media areas were assessed by image analysis (Scion image).

Thirdly, for immunostaining, another set of sections was fixed in acetone, endogenous peroxidase activity was quenched in 0.6% H₂O₂/methanol, and unspecific binding was blocked with 20% rabbit serum (DAKO, Hamburg, Germany) in PBS. A mouse monoclonal anti-Ram11 antibody (DAKO, Hamburg, Germany) was used to detect macrophages, a mouse monoclonal anti-actin antibody (Neo Markers, Fremont, CA, USA) was used to detect smooth muscle cells, and an anti-CD 43 antibody (Serotec, Oxford, UK) to detect T lymphocytes. All antibodies were applied overnight at 4°C. The sections were washed with PBS and incubated at RT with biotinylated anti-mouse-IgG second antibody (DAKO, Hamburg, Germany, 1:300) for 30 min, followed by StreptABComplex/HRP (DAKO, Hamburg, Germany, 1:100) and developed in peroxidase substrate solution (3'3'-diaminobenzidine; Liquid DAB+ Substrate Chromogen System, DAKO, Hamburg, Germany) for 6 min. After counterstaining with Harris' hematoxylin, sec-

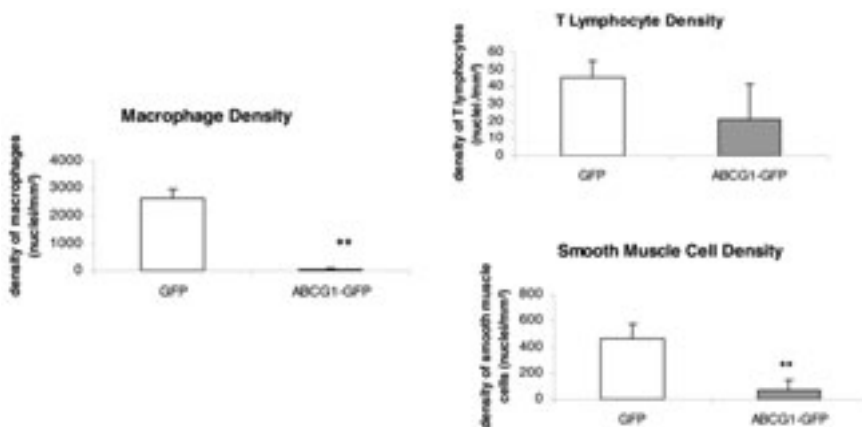


Figure 2. Quantitative assessment demonstrated a significant reduction of macrophages and smooth muscle cells, with a trend towards reduced T-lymphocyte invasion after local Ad-ABCG1-GFP gene transfer (**indicates P<0.01 compared to Ad-GFP). The means ±SEM of 6 animals are shown.

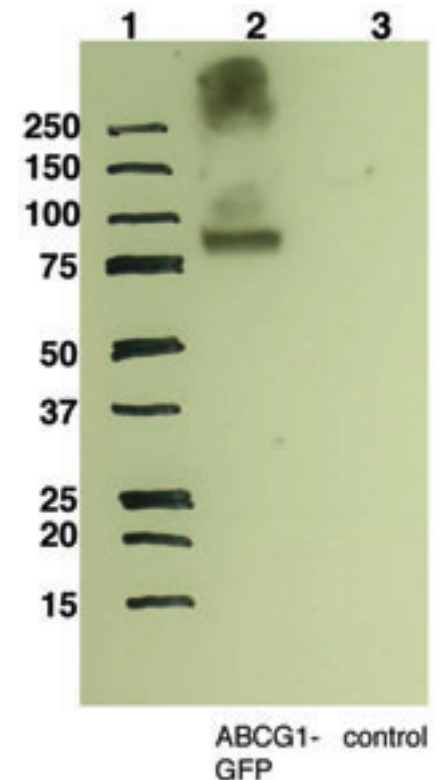


Figure 3. Transgene expression of the recombinant ABCG1-GFP fusion protein was verified by Western blotting with an anti-ABCG1 antibody, showing a clear signal at the expected size. Lane 1, protein size marker; lane 2, ABCG1-GFP; lane 3, control membrane preparation.

tions were dehydrated and mounted with Permout (Fisher Scientific, Schwerte, Germany).

Sections were viewed with a Zeiss Microscope ($\times 20$ magnification), photographed, and positive immunostaining was quantified using image analysis software (Scion image).

Quantity of immunostaining for macrophages, smooth muscles and T-lymphatic cells was determined as the number of positive cells which were identified by the presence of both immunostained brown cytoplasm and hematoxylin-counterstained blue nuclei, divided by the intima area of the stained arteries. For counting of both cells and respective areas, the whole circular area surrounding the vessel lumen of a representative section was taken into account and also studied at a higher magnification ($\times 200$). The density for each kind of cell was calculated as the ratio between the respective cell number and intima area and expressed in nucleus number \times mm² (Figure 2).

Statistical analysis

Comparisons between groups were calculated using ANOVA followed by Scheffe's *post hoc* testing for independent values using SPSS 15.0. Data represent means \pm SEM, where indicated. $P < 0.05$ was considered statistically significant.

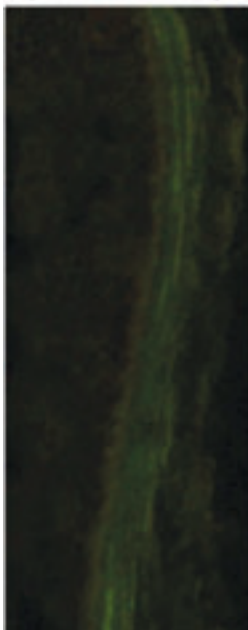
Results

Expression and function of the recombinant ABCG1-GFP fusion protein

Correctness of the sequence of ABCG1-GFP in the recombinant plasmids and viruses was checked by sequencing. The ABCG1 sequence corresponded to the sequence published under accession n. NM004915.2. After infection of HUVEC cells, Western blotting with an anti-ABCG1 antibody showed a transgene at the expected size of 95 kDa of the ABCG1-GFP fusion protein (Figure 3). Expression of the transgene coincided with green fluorescence under UV light in these cells. Addition of 10 μ mol/L of the LXR agonist all-trans retinoic acid (ATRA) resulted in an approximately 2-fold increase of the signal of ABCG1 in these cells, as assessed by semi-quantitative image analysis, whereas gene transfer with Ad-ABCG1-GFP increased the signal by approximately 10-fold.

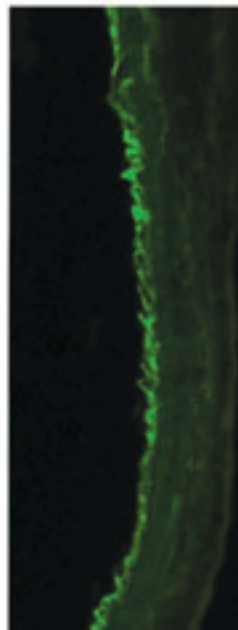
After incubation of HUVEC cells with cholesterol, specific cholesterol efflux was tested after washing and addition of HDL (100 μ g/mL). A marked increase of specific cholesterol efflux occurred from ABCG1-GFP expressing cells, which was significantly higher than from GFP only expressing control cells. The efflux was not detectable in GFP expressing

Sections of the right carotid artery (uninfected)



Sections of the left carotid artery after virus infection

Ad-GFP



Ad-ABCG1-GFP

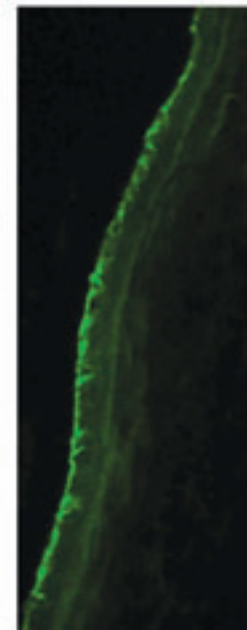


Figure 4. Expression of the reporter gene GFP was detected in the left carotid arteries of all rabbits after gene transfer *in vivo* with Ad-ABCG1-GFP (co-expressing GFP with ABCG1) and Ad-GFP. Representative histological sections show specific fluorescence in the left carotid artery after infection with Ad-GFP (middle panel) as well as after Ad-ABCG1-GFP (right panel). For comparison, only a slight background fluorescence occurred in the right carotid artery of identical rabbits without local vascular gene transfer (left panel).

Ad-GFP



Ad-ABCG1-GFP



Figure 5. Representative *en face* macroscopic preparations of left carotid arteries from atherosclerotic rabbits. Four weeks after local gene transfer with Ad-ABCG1-GFP plaque formation was vastly reduced compared to those infected with Ad-GFP only. All preparations lack a small 5 mm block in the middle which was taken out for histological analysis.

HUVEC cells which were only kept in medium and increased to $22\pm 3\%$ of total cholesterol after addition of HDL. In ABCG1 expressing cells, the basal efflux was $15\pm 2\%$, and increased to $69\pm 10\%$ after addition of HDL (results from 3 independent experiments \pm SEM, $P<0.05$).

From these experiments, we concluded that GFP expression always coincided with the presence of a functional ABCG1 transporter protein, since GFP was C-terminally fused to ABCG1, and could not have been expressed if recombinant ABCG1 had not been fully present.

GFP and ABCG1 expression in the vascular wall *in vivo*

After vascular gene transfer into the left carotid artery with either Ad-GFP or with Ad-ABCG1-GFP, GFP fluorescence could be equally detected. Figure 4 shows this reporter gene expression in the vascular wall under UV light (right panel), which is not detected in the contralateral carotid artery in identical animals (left panel). As the adenovirus expresses ABCG1 to which GFP was C-terminally fused, detection of GFP must have corresponded to the expression of recombinant ABCG1. This approach allows for a specific functional and morphological evaluation of the effects of the transgene and clearly distinguishes unspecific alterations caused by either the virus infection or the reporter gene GFP.

Animal model of atherosclerosis

All animals developed high cholesterol levels after feeding with a lipid-rich feed. There were no differences in mean serum cholesterol levels between the groups (serum cholesterol after four weeks: 2213 ± 123 mg/dL (ABCG1-GFP), 2407 ± 231 (GFP); and after eight weeks: 2732 ± 321 (ABCG1-GFP), and 2975 ± 199 (GFP)).

Four weeks after gene transfer, and eight weeks after start of cholesterol feeding, animals were investigated *in vivo* by ultrasound, then necropsied and analyzed histologically.

Effect of ABCG1 on atheroprogession

En face preparations of the left and right carotid arteries, the aortic arch and the abdominal aorta were used to determine plaque size. Observer bias was reduced by studying large overviews of different vascular sites from atherosclerosis-prone rabbits. In the region of gene transfer in the left carotid artery, relative plaque size with reference to the investigated vascular area was significantly reduced in Ad-ABCG1-GFP infected animals compared to Ad-GFP infected control rabbits (Figure 5). The mean values of the *en face* analysis of plaque extension determined by Sudan red staining is shown in Figure 6. In other vascular sites without gene transfer, the right carotid artery, or

the thoracic or abdominal aorta, which all served as controls, plaque progression was comparable between the control and the ABCG1-GFP groups.

A detailed investigation of plaque size was carried out at the same vascular sites after fine cutting in histological sections and HE and VG staining. The intima area of the left carotid artery at the site of gene transfer was markedly smaller in the Ad-ABCG1-GFP infected rabbits compared to the control Ad-GFP infected group (Figure 1). A summary of the histological analysis of intima and media areas determined after HE staining is shown in Figure 7.

Effect of ABCG1 on the cellularity of the vascular wall

The inflammatory component of atheroscle-

rosis was assessed by determination of macrophage density in the vascular wall of cholesterol-fed rabbits. Macrophage density was almost blunted after gene transfer of Ad-ABCG1-GFP to the left carotid artery of rabbits. Representative histological sections are shown in Figure 8. Also, invasion of smooth muscle cells was significantly reduced (Figure 9), whereas T-lymphocyte invasion showed a trend towards reduced values (Figure 10). The mean values of all experiments are shown in Figure 2.

Effect of ABCG1 on endothelial function

Endothelial function was determined in rabbits with vascular ultrasound *in vivo*. Flow-induced endothelial function of atherosclerotic

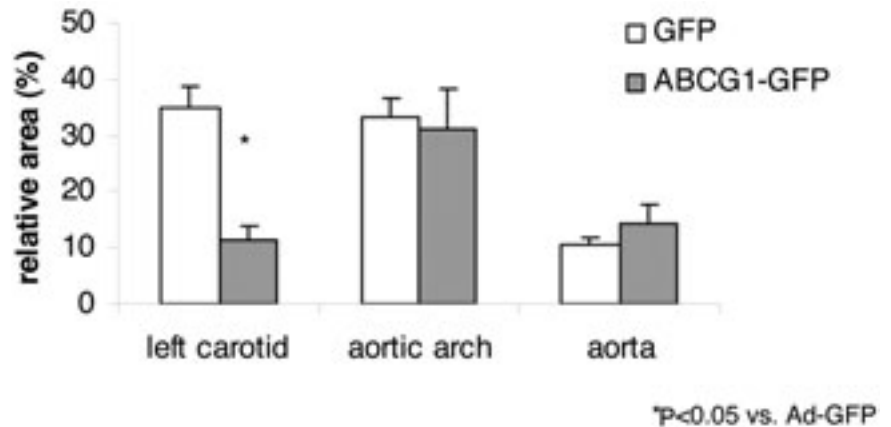


Figure 6. Quantitative assessment of plaque formation by digital image analysis showed significant reduction after local gene transfer with Ad-ABCG1-GFP. Other vascular sites without gene transfer served as internal controls demonstrating homogenous plaque progression in rabbits after high cholesterol diet. The means \pm SEM of 6 animals are shown (* indicates $P<0.05$ significance compared to Ad-GFP).

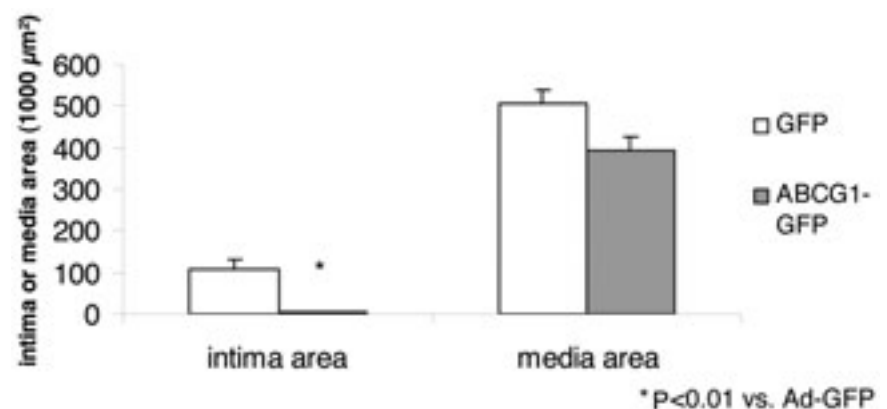


Figure 7. Quantitative assessment of intima and media areas by digital image analysis showed a marked reduction of intima area after local gene transfer with Ad-ABCG1-GFP. The means \pm SEM of 6 animals are shown (* indicates $P<0.05$ significance compared to Ad-GFP).

animals was significantly improved by vascular gene transfer with Ad-ABCG1-GFP (Figure 11). In atherosclerotic rabbits which had received Ad-ABCG1-GFP, a volume challenge led to an almost normal endothelium-mediated vasodilatation. The extent of vasodilatation observed in this model is comparable

gene transfer into the vascular wall of cholesterol-fed rabbits allows targets with therapeutic impact on the prognosis of patients with atherosclerosis to be validated.

The present study widens our understanding of the effect of an overexpression of

human ABCG1 directly in a disease background of atherosclerosis, while at the same time helping to determine the effect on endothelial function *in vivo*. Previous studies in this field have given somewhat conflicting results. Targeted disruption of ABCG1 in mice

Discussion

In this study, we demonstrate that overexpression of ABCG1 attenuated the progression of atherosclerosis in cholesterol-fed rabbits and markedly reduced inflammatory cell invasion in the vascular wall. Also endothelium-mediated vasoreactivity was improved by overexpression of ABCG1. These findings add new information to that provided by results obtained in transgenic mice. Thus, the present model of vascular gene transfer in atherosclerotic rabbits helps to judge the contribution of specific proteins to the pathophysiological process of atherosclerosis.

Cholesterol-fed rabbits are an established animal model for the investigation of atherosclerosis.¹⁹ Local somatic gene transfer into the vessel wall for the evaluation of the *in vivo* role of specific transgenes in atherosclerosis has been described previously.^{20,21} We have built up an investigation system which used effective vascular gene transfer in atherosclerotic rabbits.¹⁸ This approach allows the effect of a transgene to be studied directly in an adult disease background, and thus avoids the confounding effect of parent gene counterregulation, which often occurs in constitutively expressing transgenic mice. This is also true in the context of bone marrow transplantation studies, because the donor macrophages are also subject to life-long counterregulation. Therefore, such somatic gene transfer studies complement and add independently obtained new knowledge to the frequently used investigations in transgenic mice.

Moreover, the somatic gene transfer model allows much larger animals, such as rabbits, to be investigated and thus intravital parameters, such as endothelial function which are less reliably determined in mice, can be precisely measured. A detailed evaluation of morphological changes (post mortem studies) can be combined with previous *in vivo* functional assessment by vascular ultrasound. This kind of determination of endothelial dysfunction has been increasingly used as an early marker of atherosclerosis in both clinical studies and in clinical routine practice, and it predicts the clinical outcome in patients.²² Patients with endothelial dysfunction have a higher probability of experiencing a major cardiovascular event than those with preserved endothelial function. Therefore, this model using somatic

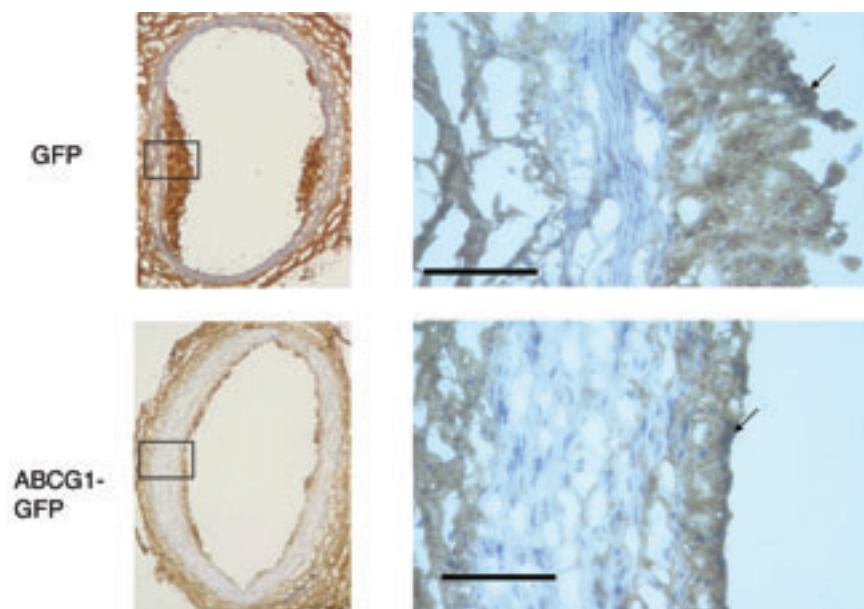


Figure 8. Representative images of cell invasion into atherosclerotic plaques, as visualized by brown staining using immunohistochemistry: macrophages. The scale bars correspond to a length of 50 μm . Positive cells are indicated by arrows.

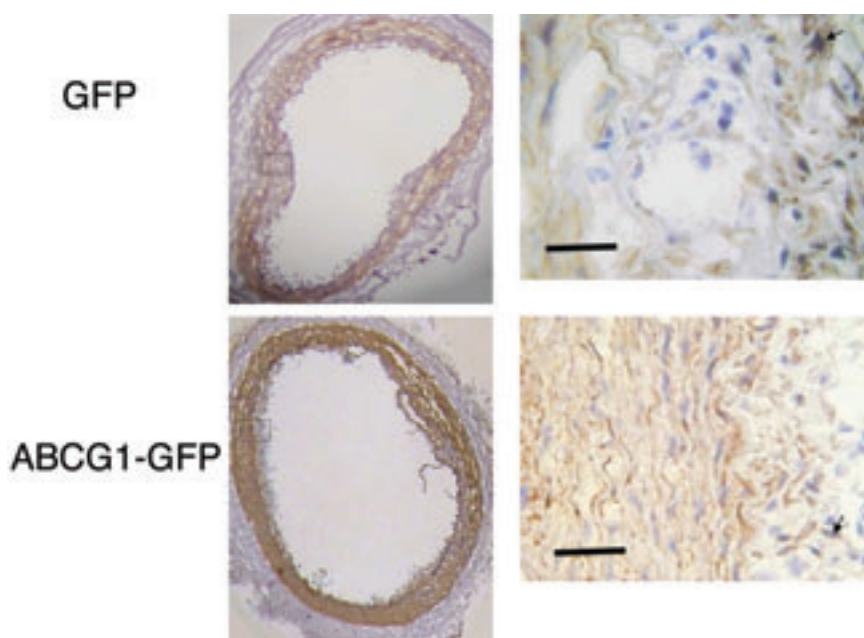


Figure 9. Representative images of cell invasion into atherosclerotic plaques, as visualized by brown staining using immunohistochemistry: smooth muscle cells. The scale bars correspond to a length of 50 μm . Positive cells are indicated by arrows.

had no effect on plasma lipids but resulted in massive lipid accumulation in hepatocytes and in macrophages. However, transplantation of the bone marrow of ABCG1-deficient mice to atherosclerosis-prone Apo E $-/-$ mice and to LDL receptor knockout mice surprisingly resulted in decreased lesion formation.^{13,14} In contrast, double knockdown of both ABCA1 and ABCG1 by transplantation of ABCA1/ABCG1 double knockout bone marrow into atherosclerosis-prone LDL receptor knockout mice resulted in substantially greater atherosclerosis than controls.¹⁵ On the other hand, contradictory findings have been published on the effects of the combined deletion of ABCA1/ABCG1 in bone marrow-derived atherosclerosis.²³

A recent study on whole-body overexpression of human ABCG1 in Apo E $-/-$ mice did not show any relevant changes of either plasma lipids or atherosclerosis.¹⁷

Macrophage deficiency of ABCG1 was associated with decreased atherosclerosis in LDLr $(-/-)$ as well as in ApoE $(-/-)$ mouse models in some studies.^{13,14} However, other studies indicated that macrophage deficiency of ABCG1 leads to moderately enhanced atherosclerosis.^{24,25} Some of these findings in transgenic mice might partly be explained by the life-long knock-out or overexpression of the transgene in all these animal models, which might have obscured the true effects by counterregulation of other ABC family members.

The present study shows that overexpression of ABCG1 *in vivo* actually attenuates atherosclerosis in a background of established atherosclerotic disease. Both plaque extension and, more importantly, plaque cellularity were markedly decreased. The clear reductions in macrophage and smooth muscle cell invasion are important indicators that the evolved plaques were probably more stable, and hence, less prone to rupture. Both parameters have been linked to the prognosis of patients in clinical studies.²² These results also confirm the concept of an impact of the cholesterol transporters ABCG1 and ABCA1 on immune responses and inflammatory activation in various tissues, as recently published in several studies.²⁵

These findings are in line with those of a recent publication which documented increased atherosclerosis in ABCG1 $-/-$ /LDLR $-/-$ double knock-out mice which were transplanted with wild-type bone marrow, compared to wild-type bone marrow-transplanted LDLr $-/-$ single knock-out mice.²⁵

This analysis has certain limitations in that we focused on the specific cells which were actually found in the plaques, so that the few cells which were found outside, in the normal endothelial lining, were not separately analyzed. In addition, we also report that an

early functional parameter of atherosclerosis, *i.e.* endothelial dysfunction, can be improved by increased ABCG1. We determined this parameter for the first time *in vivo* in a physiological measurement setting, and it agrees with another recent study of ABCG1 knockout mice which showed increased endothelial dysfunction in prepared femoral arteries *ex vivo*.²⁶ This is possibly due to the occurrence of endothelial nitric oxide synthase (eNOS) acti-

vation after HDL binding to endothelial cells which is presumably triggered by ABCG1, as recently reviewed.⁴ Since these effects may be partly due to differential regulation of endothelial adhesion proteins, it would certainly be interesting to investigate VCAM-1 expression in the vessel walls. This topic should be investigated in future studies.

Stimulation of ABCG1 might, therefore, have a beneficial effect on various stages of

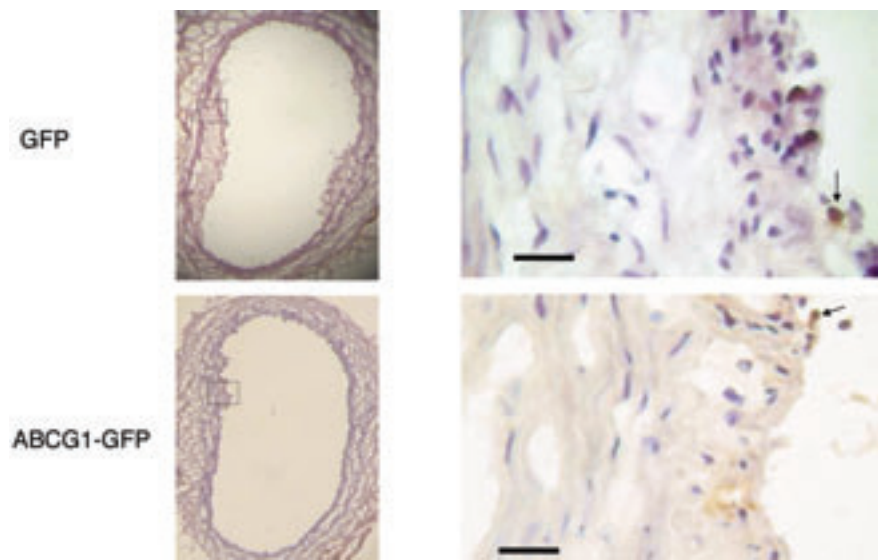


Figure 10. Representative images of cell invasion into atherosclerotic plaques, as visualized by brown staining using immunohistochemistry: T lymphocytes. The scale bars correspond to a length of 50 μ m. Positive cells are indicated by arrows.

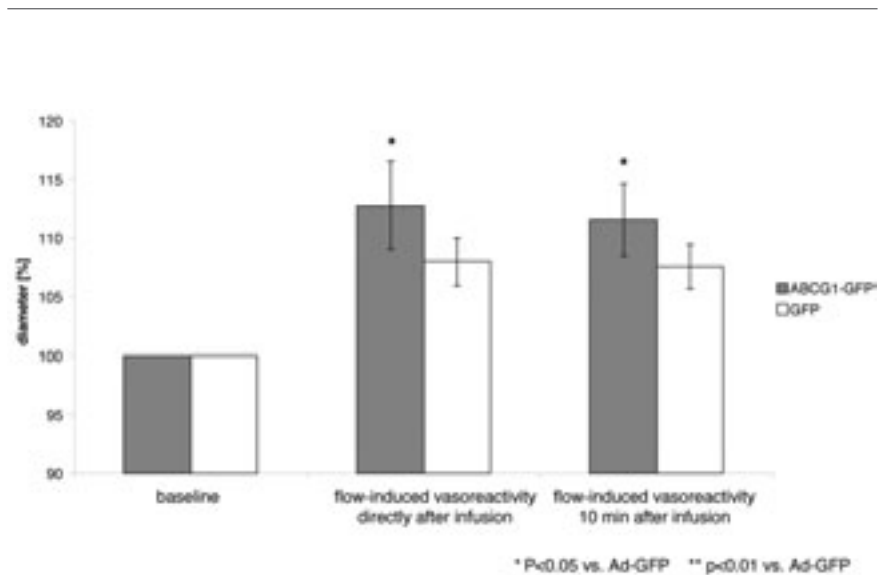


Figure 11. Endothelial dysfunction was investigated in rabbits by vascular ultrasound *in vivo* by studying the flow-induced vascular reactivity by infusion of a high volume of saline. The effect of Ad-ABCG1-GFP and of control adenovirus (Ad-GFP) in atherosclerotic rabbits was compared to the effect of Ad-GFP in healthy control rabbits. The relative change in diameter of the left carotid artery compared to baseline (%) is demonstrated after 1-2 min (directly after infusion) and 10 min after infusion. * indicates $P < 0.05$ compared to Ad-GFP-ABCG1. The means \pm SEM of at least 6 animals are shown.

atherosclerosis. As the expression of ABCG1 is induced by sterols, modified LDL, retinoid×receptor (LXR, RXR)-specific ligands, peroxisome proliferator-activated (PPAR-ligands) and IL-10, there might be several therapeutic approaches to reach that goal. Also it has been suggested that PPAR α and PPAR γ agonists inhibit the formation of macrophage foam cells and atherosclerosis in mice by upregulation of ABC transporters. LXR agonists not only stimulate the ABCG1 expression, but also the redistribution of ABCG1 from the intracellular vesicular pool to the plasma membrane in macrophages.

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