Western array analysis of human atherosclerotic plaques: downregulation of apoptosis-linked gene 2

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Received 17 March 2003; received in revised form 28 May 2003; accepted 25 June 2003

Time for primary review 21 days

Abstract

Objective: In recent years, microarray techniques have been used to characterize differences in mRNA populations between atherosclerotic plaques and normal arterial tissue. Because proteomics provide an attractive complementary approach to genomics, we used Western array technology as a global protein profiling method to identify differentially expressed proteins with potential pathobiological relevance in human atherosclerotic plaques.

Methods: Cell lysates from human carotid endarterectomy specimens and non-atherosclerotic mammary arteries were screened with monoclonal antibodies (823 in total) that were combined into unique cocktails. Hits were verified with traditional Western blotting.

Results: Seven proteins with a >5-fold relative expression difference were identified. One of the most apparent changes in human plaques was the downregulation of apoptosis-linked gene 2 (ALG-2), a positive mediator of apoptotic cell death. Differential expression of ALG-2 in human plaques relative to mammary arteries was not confirmed by real-time quantitative RT-PCR, suggesting post-transcriptional regulation. Uptake of aggregated LDL (agLDL) downregulated ALG-2 protein expression in THP-1 macrophages, but not in smooth muscle cells (SMCs). Transfection of THP-1 cells with ALG-2-specific small interfering RNA (siRNA) caused ALG-2 depletion and inhibited the execution phase of apoptosis (DNA fragmentation) but did not affect caspase-3 activation, annexin-V labeling and necrotic cell death.

Conclusion: Western array screening of carotid endarterectomy specimens revealed a strong downregulation of ALG-2 protein. Because ALG-2 has pro-apoptotic potential, our results point to a novel survival mechanism against cell death in human atherosclerotic plaques.

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Keywords: Atherosclerosis; Apoptosis; Monoclonal antibodies

This article is referred to in the Editorial by J. Herrmann in this issue.

1. Introduction

Atherosclerosis is a multifactorial disease of the arterial intima that involves several gene products including lipoprotein receptors, adhesion molecules, growth factors, cytokines, matrix metalloproteinases and proteins associated with cell death [1,2]. Recent advances in our molecular understanding of the pathogenesis of atherosclerosis have predominantly been made at the mRNA level using microarray technology [3–5]. However, investigation of diseases by analysis of RNA alone has inherent limitations, among these the analysis of the phenotype of multigenic phenomena, and the inability to predict whether gene products are translated or have undergone post-translational modifications. In addition, the correlation between mRNA levels and protein concentration is often poor. The use of proteomics-based strategies, examining the protein content of cells or tissues, is expanding rapidly and offers a powerful complementary approach, overcoming some of the limitations of RNA analysis.
Two-dimensional (2D) gel electrophoresis is currently the most popular technique for proteome analyses and has already offered much insight into the molecular mechanisms underlying cardiovascular disease [6]. Despite standardization of this technique, several technical issues remain unsolved. These issues include difficulties with isoelectric focusing and the inability to resolve all the proteins on gel (especially lower-abundance, basic and membrane proteins). Other problems are inherent to the technique itself. Indeed, 2D gel electrophoresis is very difficult to automate and requires a lot of hands-on time. Furthermore, although it is the highest resolving technique for very complex protein mixtures, multiple proteins are frequently found in one 2D gel spot so that quantitative and comparative analysis of 2D gels is prone to mistakes. Two alternative techniques that may compete with 2D gel electrophoresis are large-scale peptide or protein arrays and gel-free mass spectrometry based proteomics. In this study, we used a high-throughput Western blot analysis, also called Western array, to screen cell lysates from carotid endarterectomy specimens and non-atherosclerotic mammary arteries with 823 monoclonal antibodies. A strong downregulation of apoptosis-linked gene 2 (ALG-2) was found in advanced atherosclerotic plaques of carotid endarterectomy specimens as compared with mammary arteries. Because ALG-2 is a positive mediator of apoptosis [7], our results point to a novel mechanism inhibiting cell death in human plaques.

2. Methods

2.1. Carotid endarterectomy specimens

Human carotid endarterectomy specimens (n = 12) were obtained from patients (mean age = 72 ± 1 years, all men) with a carotid stenosis of >70% as demonstrated by digital subtraction angiography and duplex ultrasonography. The specimens were opened along their longitudinal axis. The inner wall of the distal common carotid artery, the proximal part of the external and internal carotid artery and the carotid sinus. Histological examination revealed that all specimens contained ruptured lipid-rich plaques. According to the adapted American Heart Association classification scheme as modified by Virmani et al. [8], thin fibrous cap atheromata alternated with other stages of atherosclerosis (fibrous cap atheroma and intimal xanthoma) in the same specimen. For microdissection experiments, sections (6 µm thick) of carotid endarterectomy specimens were mounted on Optiplus slides (Biogenex, San Ramon, CA) and deparaffinized in toluol. Medial smooth muscle cells (SMCs) and plaque cells were then microdissected from 10 tissue sections using a clean scalpel under direct microscopic visualization. Non-atherosclerotic mammary arteries (n = 7, mean age of patients = 72 ± 1 years, all men) obtained during bypass surgery were used as negative control samples and were manipulated similarly. All mammary artery segments showed adaptive intimal thickening and duplication of the internal elastic membrane. The investigation conforms with the principles outlined in the Declaration of Helsinki.

2.2. Antibodies

All primary antibodies were purchased from BD Biosciences Transduction Laboratories (Lexington, KY), with the exception of anti-β-actin (clone AC-15) mouse monoclonal and anti-active caspase-3 rabbit polyclonal antibody which were from Sigma (St. Louis, MO) and Cell Signaling Technology (Beverly, MA), respectively. Goat anti-mouse and sheep anti-rabbit peroxidase conjugated secondary antibodies were purchased from Jackson (West Grove, PA) and DAKO (Glostrup, Denmark), respectively.

2.3. Western array analysis

Carotid endarterectomy specimens and mammary arteries were homogenized in boiling lysis solution (10 mM Tris pH 7.4, 1 mM sodium orthovanadate, 1% SDS) and analyzed by the Powerblot™ Western array screening facility of BD Biosciences. Briefly, 300 µg of protein from each sample was loaded in one big well across the entire width of a 5–15% Criterion SDS-polyacrylamide gel (Bio-Rad, Richmond, CA). Subsequently, the gels were run for approximately 1 h at constant milliamps. After electrophoresis, the gels were transferred to Immobilon-P nylon membranes (Millipore, Bedford, MA) for 2 h at 200 mA. Membranes were blocked for 20 min with 5% milk and then inserted into a Western blotting manifold that isolates 40 channels across the membrane. In each channel, a complex antibody cocktail (3–6 antibodies) was added and allowed to hybridize for 45 min. Following staining, the blots were removed from the manifold, washed and hybridized for 30 min with secondary peroxidase-conjugated goat anti-mouse antibody. All primary antibodies were mouse monoclonal. Membranes were washed and developed using Supersignal West Dura Extended Duration Substrate (Pierce, Rockford, IL). Digitally captured images of all blots were subjected to automatic spot finding and spot matching using PDQuest software (Bio-Rad). Expression data were normalized by dividing the signal obtained for each protein by the sum of signals obtained for all examined proteins for one given sample. To define differential protein expression, we used a 5-fold threshold value. Western array reproducibility was determined using three independent assays.
2.4. Immunohistochemistry

Immunohistochemical reactions were carried out by an indirect peroxidase antibody conjugate method as described [9].

2.5. Real time quantitative RT-PCR

Relative abundance of ALG-2 mRNA was assessed using the 5′ fluorogenic nucleic assay (TaqMan) on an ABIPrism 7700 sequence detector system (Applied Biosystems, Foster City, CA). Human ALG-2 PCR primers 5′-ATCGCCCTTC-GACGACTTCAT-3′ (forward) and 5′-GACCATGGA-CAGGTACTTCTGTA-3′ (reverse) as well as the fluorogenic probe 5′-ATCCGTCACCTCTGACGAGT-3′ were designed using Primer Express software (Applied Biosystems). The probe was 5′-FAM (reporter) and 3′-TAMRA (quencher) labeled.

Total RNA was prepared from tissue sections or cultured cells using the QIAamp Viral RNA Mini Kit (Qiagen, Chatsworth, CA) and the Absolutely RNA Nanoprep Kit (Stratagene, La Jolla, CA), respectively. All RNA samples were treated with RNase-free DNase I (Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed in duplicate in 25 μl reaction volumes consisting of 1 × Master Mix and 1 × Multiscribe and RNase inhibitor Mix (TaqMan One Step PCR Master Mix Reagents Kit, Applied Biosystems). PCR cycling parameters were: reverse transcription at 48 °C for 30 min, inactivation of RT at 95 °C for 10 min, followed by 40 cycles consisting of incubations at 95 °C for 15 s and 60 °C for 1 min. Relative expression of mRNA species was calculated using the comparative Ct method. All data were controlled for quantity of RNA input by performing measurements on the endogenous reference gene β-actin (Taqman β-actin detection reagent, Applied Biosystems).

2.6. Cell culture

The human THP-1 monocyte cell line (American Type Culture Collection) was grown in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamycin (50 μg/ml) in an atmosphere of 95% air and 5% CO2. Cells were differentiated into macrophages by the addition of 0.2 μM phorbol 12-myristate 13-acetate (PMA, Sigma) to the medium for 24 h. Human aortic smooth muscle cells (SMCs) (American Type Culture Collection) were grown in smooth muscle cell basal medium (Cambrex, East Rutherford, NJ) supplemented with human recombinant epidermal growth factor (0.5 ng/ml), insulin (5 μg/ml), human recombinant fibroblast growth factor (2 ng/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), amphotericin-B (50 ng/ml) and 5% fetal bovine serum. To induce foam cell formation, THP-1 macrophages were incubated with aggregated LDL (agLDL, Sigma, 50 μg/ml) for up to 48 h in medium containing 10% lipoprotein-deficient serum (Sigma) and antibiotics (vide supra). Since lipid uptake in SMCs is less efficient in macrophages, SMCs were exposed to 300 μg/ml agLDL for up to 3 days. Lipid peroxidation of LDL as determined by measuring thiobarbituric acid reactive substances was low and always approximated the detection limit of the assay (~ 1 μM of malondialdehyde equivalents). agLDL was made by vortexing the LDL solution for 4 min in Eppendorf tubes. In some experiments, LDL was oxidized with CuCl2 as previously described [10]. To analyze lipid uptake, cells were fixed in 4% formaldehyde (10 min) and stained with oil red O.

2.7. ALG-2 gene silencing and cell death determination

Small interfering RNA (siRNA) specific to ALG-2 was synthesized with the Silencer™ siRNA Construction Kit (Ambion, Austin, TX) using siRNA oligonucleotide templates 5′-AAAGACAGGAGTGGATGATACCTGTCCTC-3′ and 5′-AATATACAATCTCTCAGCTC-3′. Oligonucleotides 5′-AAGAGATGGAGTAACGGTAGGC-3′ and 5′-AATACCGTACTCTA-CCTTCTCTC-3′ were used as scrambled control templates. To induce gene silencing, THP-1 cells were plated at 106 cells per well in a 6-well tissue culture plate in the presence of 0.2 μM PMA and transfected 24 h later with 40–100 nM ALG-2 siRNA or control siRNA in combination with siPORT™ Lipid transfection agent using the Silencer™ siRNA Transfection Kit (Ambion). For some experiments, siRNA was labeled with Cy3 using the Silencer™ siRNA Labeling Kit (Ambion). Apoptotic cell death of siRNA-transfected cells was detected by flow cytometry using an Annexin V-Propidium Iodide Kit (BD Biosciences) and an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany).

2.8. Statistical analysis

The results are expressed as mean ± S.E.M. The 95% confidence interval for the relative expression of ALG-2 verified by real time quantitative RT-PCR was calculated by the unpaired Student t-test. To test whether the relative expression of ALG-2 in agLDL-treated SMCs and THP-1 macrophages was different from the untreated control group, the one sample t-test was used with a specific constant of 100%. A value of P<0.05 was considered significant.

3. Results

3.1. Comparative Western array analysis

Crude protein extracts were prepared from pooled carotid endarterectomy specimens and from segments of non-
atherosclerotic mammary arteries. Proteins were then analyzed by Western array technology using 823 different antibodies which are mainly directed against signal-transducing proteins. When we analyzed data from three independent runs, the Western arrays showed a highly reproducible pattern of protein expression (<2% of proteins with 5-fold difference in gene expression between the different runs). We obtained good quality signals (signal intensity >3000 and with well-defined boundaries) for approximately 14% of the examined protein targets (Fig. 1). Initial examination of the array patterns led to the identification of 15 proteins that were differentially expressed (>5-fold difference in signal intensity in triplicate) between normal and diseased tissue. To validate array results, expression of the 15 selected proteins was analyzed by standard immunoblot assays (one antibody per blot). In this way, only 7 of the 15 proteins showed differential protein expression (Fig. 2), suggesting a high rate of false-positive signals. Thrombospondin-2 (TSP-2), manganese superoxide dismutase (MnSOD), apolipoprotein B-100 (apoB100), protein-tyrosine phosphatase 1C (PTP1C) and apolipoprotein E (apoE) were significantly upregulated in the endarterectomy specimens, whereas apoptosis-linked gene 2 (ALG-2) and glycogen synthase kinase-3β (GSK-3β) were downregulated.

To rule out the possibility that differential expression in carotid plaques vs. mammary arteries results from a different cell type composition, signal intensity of the seven differentially expressed proteins was examined by standard Western blotting in human aortic SMCs and THP-1 macrophages (before and after differentiation with phorbol 12-myristate 13-acetate) (Fig. 2). TSP-2, MnSOD and apoE were preferentially expressed in SMCs whereas PTP1C was only detectable in macrophages (regardless of the differentiation status). Expression of ALG-2 and GSK-3β was similar in SMCs and macrophages. ApoB100 could not be detected, neither in SMCs nor in macrophages.

3.2. Expression of ALG-2 in human atherosclerosis

Because we were mostly interested in proteins that are associated with apoptotic cell death, we focused our further...
analysis on ALG-2 expression. In contrast with the Western data, real time quantitative RT-PCR did not show a significant difference of ALG-2 expression (1.2-fold upregulation in carotid plaques vs. mammary arteries, 95% confidence interval = 0.43–3.39), indicating that ALG-2 expression is mainly regulated at the post-transcriptional level. Using the same antibody for Western blotting, both carotid endarterectomy specimens and mammary arteries did not show a clear immunohistochemical staining for ALG-2 (Fig. 3A–B). However, immunohistochemical staining of squamous cell carcinoma of lung tissue showed a strong positive reaction in the tumor cells, both in the cytoplasm and the nucleus, in contrast to adjacent healthy tissue which was negative (Fig. 3C). This could indicate that ALG-2 expression levels are low (and thus difficult to detect by immunohistochemistry) in normal tissue, as recently reported [11], but that they may significantly increase in tumor cells.

Fig. 3. Immunohistochemical detection of ALG-2. Smooth muscle cells of mammary arteries (A) and atherosclerotic plaques (B) show a weak signal for ALG-2. In contrast, an abundant nuclear and cytoplasmic signal was present in squamous cell carcinoma of the lung (C). Scale bar = 50 μm.

Fig. 4. Microdissection of plaque area and adjacent inner media from tissue sections of a whole-mount carotid endarterectomy specimen and analysis of ALG-2 expression in the microdissected regions via Western blotting. The figure shows an example of a tissue section before (A) and after (B) microdissection. Sections were stained with hematoxylin and eosin (panel A) or hematoxylin (panel B). The specimen contained the inner wall of the distal common carotid artery (cc), the bifurcation with the sinus caroticus (sc), the arteria carotis interna (ci) and externa (ce). The specimen also contained remnants of the inner media (M) that was cleaved during the surgical procedure in the communis region and at the flow divider. These fragments of adjacent media as well as a large plaque (PL) area (delineated in panel A) were microdissected and further analyzed for expression of ALG-2 via Western blotting (C). Scale bar = 5 mm.
In an additional experiment, plaque cells as well as SMCs present in adjacent margins of normal media were micro-dissected from the same carotid endarterectomy specimen and subsequently analyzed for expression of ALG-2 via Western blotting (Fig. 4). ALG-2 was detectable in medial smooth muscle cells, but not in cells that were procured from the adjacent plaque. Since accumulation of intracellular lipid (foam cell formation) is a prominent feature of cells present in human atherosclerotic plaques [2], we examined whether foam cell formation could influence ALG-2 expression. For this purpose, human aortic SMCs and THP-1 macrophages were cultured either in the presence or absence of agLDL. Initially, both cell types did not contain significant amounts of lipid as shown by oil red O staining, but slowly transformed into foam cells when exposed to agLDL. Maximal uptake of agLDL in SMCs and THP-1 cells could be obtained after 72 and 12–24 h, respectively. THP-1 macrophages clearly downregulated ALG-2 protein expression after 48 h of treatment (Fig. 5A), suggesting that foam cell formation influences ALG-2 expression and not vice versa. However, foam cell formation did not alter ALG-2 mRNA levels (not shown). Downregulation of ALG-2 was also noticed if THP-1 cells were treated with aggregated oxLDL. SMCs did not show significant differences in ALG-2 protein expression, even after 4 days of treatment (Fig. 5B).

3.3. ALG-2 silencing protects macrophages against apoptotic cell death

Since ALG-2 is essential for the execution of apoptosis by various signals [7], downregulation of ALG-2 in macrophages may protect cells against cell death. To test this assumption, small interfering RNA (siRNA) specific for ALG-2 was introduced in THP-1 cells so that ALG-2 gene silencing may occur via RNA interference (RNAi). Experiments with Cy3-labeled siRNA indicated that only a small amount (<20%) of THP-1 cells internalized fluorescent siRNA after transfection. Therefore, we decided to sort out first siRNA-positive cells by flow cytometry before ALG-2 silencing was evaluated via Western blotting. As shown in Fig. 6A, ALG-2 expression was efficiently repressed 72 h after transfection in cells containing ALG-2-specific siRNA compared to cells that were transfected with control siRNA (same nucleotide composition as ALG-2 siRNA but non-homologous to human genome). Shortly after adding the transfection agent/siRNA complexes to the cells (2–4 h), we observed spontaneous cell death (increased oligonucleosomal DNA fragmentation and propidium iodide labeling) in comparison with cells that were only treated with the transfection agent. Moreover, cells became fragile and were difficult to manipulate (spontaneous cell lysis during consecutive centrifugation steps or laser excitation). This suggests that uptake of any siRNA is toxic for THP-1 macrophages, even at a concentration as low as 40 nM. However, a major fraction of transfected cells survived siRNA uptake. Seventy-two hours after transfection, flow cytometric analyses of the latter showed a clear down-regulation of spontaneous oligonucleosomal DNA fragmentation (TUNEL) in ALG-2 siRNA-transfected cells (Fig. 6B) as compared with cells that were transfected with control siRNA. In contrast to TUNEL, there was no significant difference in annexin-V binding, cleavage of caspase-3 or propidium iodide labeling (Fig. 6C–D). If 7-ketocholesterol (100 μM) was added to the culture medium 16 h before measurement of cell death parameters, TUNEL reactivity of ALG-2 siRNA-transfected cells was similar to the control group, suggesting that resis-
stance against cell death collapsed by additional apoptotic stimuli.

4. Discussion

Powerful high throughput proteome methodologies such as 2D gel electrophoresis may show considerable promise in the identification of new gene products that influence development of atherosclerosis. Therefore, we used for the first time in cardiovascular research Western arrays to simultaneously analyze relative changes in the expression levels of hundreds of proteins (analogous to changes in mRNA levels with DNA microarray technology) in carotid endarterectomy specimens and non-atherosclerotic mammary arteries. A Western array screening of total cell or tissue lysates includes polyacrylamide gel electrophoresis and Western blotting followed by screening of the blots with monoclonal antibodies that are combined into unique cocktails. Because each monoclonal antibody identifies a unique target among the array of thousands of proteins displayed on the Western blot, this approach of proteome analysis eliminates the need for further protein identification such as necessary with 2D gel electrophoresis. At present, the number of reports that utilize this type of technology is limited [12–15], most likely because the technique has only recently been developed, but may increase rapidly as it is time saving and highly sensitive (detection of proteins at the nanogram level). It should be noted, however, that our Western array experiments revealed a high rate of false-positive results. Among the 15 proteins that were differentially expressed (>5-fold) in carotid plaques vs. mammary arteries, only seven proteins could be confirmed by standard immunoblot assays (one antibody per blot). False-positive protein spots on Western arrays may result from proteolytic degradation of the protein target during sample preparation so that fragments of a specific protein may overlap with proteins of lower molecular weight. Alternatively, antibodies may cross-react with non-specific proteins. We therefore believe that all Western array results should be confirmed by additional Western blots and that a threshold value of at least 5-fold must be used to minimize false-positive signals.

Western array screening of human carotid endarterectomy specimens and non-atherosclerotic mammary arteries revealed seven proteins with a >5-fold relative expression.

Fig. 6. Silencing of ALG-2 expression by RNA interference in THP-1 macrophages. (A) THP-1 cells were transfected with Cy3-labeled siRNA to ALG-2 or with Cy3-labeled control siRNA (same nucleotide composition as ALG-2 siRNA but non-homologous to human genome). After culturing cells for 72 h, cells that contained siRNA were sorted out by flow cytometry. The amount of ALG-2 and cleaved caspase-3 was then determined by immunoblotting. β-actin served as a loading control. (B–D) Determination of different cell death parameters by flow cytometry 72 h after transfection on the total pool of transfected cells. Shaded and open histograms represent ALG-2 siRNA and control siRNA transfected cells, respectively.
Infiltrates into the vessel wall during lesion formation [2]. In human plaques, is an important component of LDL that is somewhat abundant in human plaques, is an important component of LDL that is related to normal vessels and support the identification of apoptosis-related genes newly associated with atherosclerosis. One of the most interesting changes associated with atherosclerotic plaques in carotid arteries is the downregulation of ALG-2, a recently discovered pro-apoptotic protein that belongs to a Ca^{2+}-binding protein family with EF-hand motifs [7,18,19]. Although it is unclear how ALG-2 affects apoptosis, evidence indicates that ALG-2 becomes rapidly associated with ALG-2-interacting protein-1 in a Ca^{2+}-dependent manner [20,21]. ALG-2 also binds to the death domain of Fas and dissociates from Fas during Fas-mediated apoptosis [22]. More recently, it has been demonstrated that ALG-2 interacts with apoptosis signal-regulating kinase 1 (ASK1) which results in the nuclear presence of ASK1 and ASK1-dependent inhibition of c-Jun N-terminal kinase (JNK) activity [23]. Apoptosis has been identified as a prominent feature of advanced human atherosclerotic plaques [24–27]. Although many gene products are involved in cell death commitment pathways underlying atherogenesis, the number of apoptosis-related genes that show differential gene expression at the mRNA level is moderate as recently shown by cDNA expression arrays [5]. This finding indicates that most cell death proteins are regulated via post-transcriptional mechanisms including protein–protein interactions, post-translational modifications (e.g. phosphorylation, proteolytic cleavage, glycosylation) and regulation of their subcellular localization which cannot be detected with cDNA arrays. It also confirms the urgent need for additional proteomic-based studies to overcome this particular limitation of RNA analysis. Indeed, in the present study, we could not detect downregulation of ALG-2 by real time quantitative RT-PCR. Cell culture experiments showed that macrophages, unlike smooth muscle cells, downregulate ALG-2 if they are treated with agLDL. Since previous reports have shown that macrophages develop several anti-apoptotic mechanisms by uptake of oxLDL or agLDL [25], it is likely that downregulation of ALG-2 represents a novel anti-apoptotic strategy of macrophages in human plaques. To address this question, we tried to silence ALG-2 expression by RNA interference (RNAi) in THP-1 macrophages. RNA interference or post-transcriptional gene silencing is a term describing the specific suppression of genes by introducing complementary double-stranded RNA intracellularly [28]. Recently, it has been demonstrated that 21 base double-stranded RNA (small interfering RNA or siRNA) is a potent mediator of the RNAi effect in mammalian cells [29]. Transfection of ALG-2-specific siRNA in macrophages downregulated ALG-2 protein expression as shown by Western blot analysis of transfected cells and reduced oligonucleosomal DNA fragmentation (TUNEL reactivity) characteristic of the execution phase of apoptotic cell death. However, ALG-2 depletion did not influence activation of caspase-3 or flippage of phosphorylaser to the outer membrane, suggesting that ALG-2 does not interfere with early stages of apoptosis. Lacanà et al. [18] observed similar effects in the mouse T cell hybridoma 3DO after ALG-2 depletion by antisense RNA (activation of ICE/Ced-3 proteases, PARP cleavage and reduced DNA fragmentation) rendering ALG-2-depleted 3DO clones resistant against several cell death stimuli, including synthetic glucocorticoids, TCR and Fas triggering [7]. These findings suggest that ALG-2 exerts its function at a distal step in the apoptotic program (downstream of caspase activation) but common to different cell death pathways. Interestingly, Nhan et al. [30] suggested that macrophages in atherosclerotic plaques exist in an ‘undeath’ or ‘death-primed’ state because they contain cleaved caspase-3 as well as the short form of PARP-1 (85 kDa), a classic caspase-3 substrate, but do not show TUNEL reactivity. Although not proven, it is possible that these cells are protected against apoptosis by downregulating ALG-2. Of note, ALG-2 is not involved in necrotic cell death as ALG-2 depletion did not affect propidium iodide incorporation. Previous reports have demonstrated that when apoptosis is blocked, at least in some cell types, an alternative, slower process leading to necrosis becomes apparent [31]. Therefore, it is plausible that ALG-2-deficient cells in human plaques eventually die via another route (necrotic-like) and contribute to the formation of a central necrotic core, one of the hallmarks of an advanced atherosclerotic lesion. It is also important to note that ALG-2, next to its involvement in apoptotic processes, may play a role during cell proliferation. Indeed, Krebs et al. [11,32] demonstrated that ALG-2 is significantly amplified in various tumors of human origin, especially in metastatic cells, and translocates to the nucleus during the cell cycle. They propose that ALG-2 should be regarded as an important modulator at the interface between cell proliferation and cell death by interacting with a variety of different targets. Clearly, more work is required to elucidate the mechanisms underlying ALG-2-mediated cell death and/or the potential involvement of ALG-2 in cell proliferation.

In conclusion, we found a strong downregulation of the pro-apoptotic protein ALG-2 in human plaques which may point to a unique anti-apoptotic mechanism. As the mechanisms responsible for macrophage resistance to apoptosis and persistence in pathological conditions are poorly understood, this finding may shed some light on how macrophage-derived foam cells survive pro-apoptotic signaling in human plaques. Interestingly, ALG-2 depletion could not be demonstrated at the mRNA level, suggesting that proteomic-based strategies are needed to fully understand the pathogenesis of atherosclerosis in molecular terms.
Acknowledgements

Research was supported by the Fund for Scientific Research–Flanders (projects G.0080.98 and G.0180.01). MM Kockx held a fund for fundamental clinical research of the Fund for Scientific Research–Flanders. The authors are indebted to Martine De Bie and Jeff Thielemans for the excellent technical assistance.

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