ATG16L1 Expression in Carotid Atherosclerotic Plaques Is Associated With Plaque Vulnerability

Joelle Magné, Peter Gustafsson, Hong Jin, Lars Maegdefessel, Kjell Hultenby, Annika Wernerson, Per Eriksson, Anders Franco-Cereceda, Petri T. Kovanen, Isabel Gonçalves,* Ewa Ehrenborg*

Objective—Autophagy has emerged as a cell survival mechanism critical for cellular homeostasis, which may play a protective role in atherosclerosis. ATG16L1, a protein essential for early stages of autophagy, has been implicated in the pathogenesis of Crohn’s disease. However, it is unknown whether ATG16L1 is involved in atherosclerosis. Our aim was to analyze ATG16L1 expression in carotid atherosclerotic plaques in relation to markers of plaque vulnerability.

Approach and Results—Histological analysis of 143 endarterectomized human carotid atherosclerotic plaques revealed that ATG16L1 was expressed in areas surrounding the necrotic core and the shoulder regions. Double immunofluorescence labeling revealed that ATG16L1 was abundantly expressed in phagocytic cells (CD68), endothelial cells (CD31), and mast cells (tryptase) in human advanced plaques. ATG16L1 immunogold labeling was predominantly observed in endothelial cells and foamy smooth muscle cells of the plaques. ATG16L1 protein expression correlated with plaque content of proinflammatory cytokines and matrix metalloproteinases. Analysis of Atg16L1 at 2 distinct stages of the atherothrombotic process in a murine model of plaque vulnerability by incomplete ligation and cuff placement in carotid arteries of apolipoprotein-E-deficient mice revealed a strong colocalization of Atg16L1 and smooth muscle cells only in early atherosclerotic lesions. An increase in ATG16L1 expression and autophagy flux was observed during foam cell formation in human macrophages using oxidized-LDL.

Conclusions—Taken together, this study shows that ATG16L1 protein expression is associated with foam cell formation and inflamed plaque phenotype and could contribute to the development of plaque vulnerability at earlier stages of the atherogenic process. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.114.304840.)

Key Words: ATG16L1 ■ atherosclerosis ■ autophagy ■ carotid plaque
atherosclerotic plaques with high risk for causing events are considered to be vulnerable or rupture-prone. These vulnerable plaques are rich in lipids and inflammatory infiltrates and have thin fibrous caps poor in smooth muscle cells (SMC).\(^7\) The high-risk thin-cap fibroatheromas easily rupture and suddenly precipitate local thrombosis, which is the root cause of the clinically significant atherothrombotic complications of the disease, notably myocardial infarction and stroke.

Analyses of autophagy-defective animal models have revealed that autophagy plays a protective role during early atherosclerosis.\(^8\) In Ldr knockout mice, a macrophage-specific deletion of Atg5 has shown to increase atherosclerotic lesion size with an expansion of the necrotic core.\(^9\) In apolipoprotein-E knockout mice, proatherogenic inflammasome activity has been associated with defective autophagy in macrophages.\(^10\) It has recently been demonstrated that autophagy is also involved in the clearance of lipid droplets in macrophages, thus contributing to cholesterol efflux in macrophage foam cells.\(^11\) Indeed, studies conducted by De Meyer and colleagues showed that autophagy can be specifically stimulated in murine macrophages by drugs, such as everolimus, an inhibitor of mammalian target of rapamycin or imiquimod, a toll-like receptor 7 ligand.\(^12,13\) Interestingly, implementation of second-generation eluting stents coated with mammalian target of rapamycin inhibitors has shown to reduce the incidence of restenosis and thrombosis after angioplasty.\(^14\)

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In the present work, we studied whether a protein involved in the early steps of the autophagy process is expressed in advanced human atherosclerotic plaques by characterizing the expression of the autophagy protein ATG16L1 in carotid atherosclerotic plaques. Additionally, we analyzed whether ATG16L1 is related to plaque vulnerability by assessing plaque inflammation, as well as histological and biochemical markers characteristic of vulnerable plaque phenotype. To further define whether ATG16L1 could be involved in plaque vulnerability at different stages of the athero-thrombotic process, analyses of ATG16L1 expression were performed at 2 distinct stages of the plaque progression in a murine model of plaque vulnerability induction by incomplete ligation and subsequent cuff placement in carotid arteries from apolipoprotein-E-deficient mice. Because apoptosis via caspase-3 activation recently has been shown to directly induce ATG16L1 degradation and autophagy impairment in Crohn’s disease,\(^17,18\) we sought to determine whether ATG16L1 protein expression as well as autophagy flux could be modulated in human primary macrophages during foam cell formation using oxidized low-density lipoprotein (oxLDL) as an early atherogenic stimulus known to trigger lipid storage, inflammatory response, and apoptosis.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### ATG16L1 in Human Advanced Carotid Artery Plaques

To determine the extent of ATG16L1 expression in carotid atherosclerotic plaques, 143 human carotid plaques were analyzed first by immunohistochemistry. As controls, 5 human mammary arteries free of atherosclerosis were also analyzed. The clinical characteristics of the patients are summarized in Table 1. In carotid atherosclerotic plaques, ATG16L1 was expressed abundantly in the shoulder regions, areas surrounding the necrotic core, and healed ruptured regions, whereas in healthy mammary arteries free of atherosclerosis, ATG16L1 showed a homogenous expression across the vessel wall (Figure 1). There were no significant differences in the area stained for ATG16L1 between plaques from symptomatic and asymptomatic patients. No significant differences were found in the area stained for ATG16L1 when comparing patients with or without clinical risk factors, such as diabetes mellitus, dyslipidemia, hypertension, obesity, or statin treatment. The area stained for ATG16L1 correlated positively with the area stained for lipids (Oil Red O staining; \(r=0.341, P<0.005\)) and for phagocytic cells, such as macrophages (CD68 staining; \(r=0.455; P<0.005\), Table 2). A significant negative association was found between ATG16L1 and the SMC marker, \(\alpha\)-actin (\(r=−0.261, P<0.01\); Table 2). The above data show that in advanced atherosclerotic plaques, ATG16L1 is associated with plaques rich in lipids (necrotic core) and with.

#### Table 1. Clinical Characteristics of the Patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>N</td>
<td>143</td>
</tr>
<tr>
<td>Age, y</td>
<td>70 (SD, 9.5)</td>
</tr>
<tr>
<td>Body mass index</td>
<td>26.8 (SD, 3.9)</td>
</tr>
<tr>
<td>Male/Female</td>
<td>96/47</td>
</tr>
<tr>
<td>Degree of stenosis, %</td>
<td>90 (IQR 75–95)</td>
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<tr>
<td>Diabetes mellitus, %</td>
<td>55</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>75</td>
</tr>
<tr>
<td>Smoking (in the past or currently), %</td>
<td>80</td>
</tr>
<tr>
<td>Dyslipidemia, %</td>
<td>97</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.3 (IQR 3.5–5.1)</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.3 (IQR 1.8–3.4)</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.1 (IQR 0.9–1.4)</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.3 (IQR 0.9–1.9)</td>
</tr>
<tr>
<td>High-sensitive CRP, mg/L</td>
<td>3.7 (IQR 2.2–7.4)</td>
</tr>
<tr>
<td>Statins, %</td>
<td>88</td>
</tr>
</tbody>
</table>

Values are mean±SD or median±interquartile range or count as indicated. HDL indicates high-density lipoprotein; and LDL, low-density lipoprotein.
in intracellular lipids (macrophage foam cells), but poor in contractile SMCs, all characteristics of a vulnerable plaque.

ATG16L1 Expression, Inflammation, and Extracellular Matrix Proteins

To estimate whether ATG16L1 could be related to inflammatory pathways and matrix degrading proteins involved in the destabilization of the atherosclerotic plaques, correlation analyzes were performed. ATG16L1 expression, measured histologically, was analyzed looking for possible associations to plaque contents of cytokines and matrix metalloproteinases (MMPs), measured by ELISA. ATG16L1 expression area correlated positively with plaque contents of cytokines and chemokines, namely interleukin-6, monocyte chemoattractant protein-1, macrophage inflammatory protein-1β, regulated on activation T-cells expressed and secreted and chemokine receptor 7 (Table 2). Additionally, plaque expression of ATG16L1 correlated with plaque content of MMP1, MMP2, MMP9, and MMP10 (Table 2).

Colocalization of ATG16L1 and Cell Type Markers in Human Carotid Plaques

Transmission electron microscopy micrographs of human carotid atherosclerotic plaques have revealed structures identified as autophagosomes in disintegrating SMCs in the fibrous cap. Previous animal studies have suggested a contribution of macrophage autophagy to the atherogenic process. Therefore, in the present study, we sought to investigate which cell types are the sources of ATG16L1 in human plaques. Double immunofluorescence staining for ATG16L1 and cell type markers showed a relative colocalization between ATG16L1 and SMC-α-actin. ATG16L1 was abundantly expressed in phagocytic cells, endothelial cells (EC), and mast cells in areas surrounding intraplaque microvessels as revealed by colocalization analysis with CD68, CD31, and tryptase, respectively (Figure 2).

To further identify the cell types expressing ATG16L1 in human atherosclerotic plaques, immuno-electron microscopy studies were performed. In parallel to ATG16L1 immunogold labeling, staining of the ultrathin sections for CD68, α-SMC-actin, and CD31 was performed to facilitate the location of intraplaque macrophages, SMCs, and ECs. Occasional

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**Table 2. Significant Spearman Correlations Between Plaque ATG16L1, Plaque Histology, Plaque Cytokines and Chemokines, and Plaque MMPs**

<table>
<thead>
<tr>
<th>Plaque Histology</th>
<th>α-Actin</th>
<th>CD68</th>
<th>Oil red 0</th>
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<tbody>
<tr>
<td>r</td>
<td>−0.261*</td>
<td>0.341†</td>
<td>0.455†</td>
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<tr>
<th>Plaque Cytokines and Chemokines</th>
<th>IL-6</th>
<th>MCP-1</th>
<th>MIP-1β</th>
<th>RANTES</th>
<th>CCR7</th>
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<tbody>
<tr>
<td>r</td>
<td>0.280†</td>
<td>0.296†</td>
<td>0.241*</td>
<td>0.249†</td>
<td>0.458*</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Plaque MMPs</th>
<th>MMP1</th>
<th>MMP2</th>
<th>MMP9</th>
<th>MMP10</th>
</tr>
</thead>
<tbody>
<tr>
<td>r</td>
<td>0.306†</td>
<td>0.219‡</td>
<td>0.299†</td>
<td>0.259†</td>
</tr>
</tbody>
</table>

*CCR7 indicates cell chemokine receptor 7; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MIP-1β, macrophage inflammatory protein-1β; MMP: matrix metalloproteinases; and RANTES, regulated on activation T-cells expressed and secreted.

*P<0.01.
†P<0.005.
‡P<0.05.

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**Figure 1.** Representative micrographs of ATG16L1 staining (brown) in frozen sections of carotid atherosclerotic plaques and in frozen section of healthy mammary artery are shown at low magnification (scale bar=500 μm). Boxed areas in carotid atherosclerotic plaques depict fibrous cap, shoulder, necrotic core, and ruptured plaque regions at higher magnification (scale bar=50 μm). L indicates lumen; NC, necrotic core; and Thr, thrombus.
cytoplasmic ATG16L1 labeling of macrophages in the carotid plaques was observed (Figure 3A). Abundant labeling of ATG16L1 was observed in the cytoplasm and in areas close to membrane-bound organelles in foamy SMCs and ECs (Figure 3B and 3C).

Atg16L1 in Vulnerable Murine Carotid Plaques
Analyses of Atg16L1 expression were performed in an atherosclerotic carotid plaque vulnerability model in apolipoprotein-E-deficient mice and contralateral control carotid arteries of the same mice. Approximately 50% of the 16-week-old male mice display features of vulnerable plaques, such as intraluminal thrombus formation as observed by hematoxilin-eosin staining (Figure 4A). To better understand whether Atg16L1 could be involved in earlier phases of the atherogenic process, carotid arteries obtained from this murine model of vulnerable plaques were analyzed by immunofluorescence at 2 distinct stages of the plaque progression. Atg16L1 expression was augmented in carotid arteries developing intimal hyperplasia (stable fibrous cap) as compared with uninjured healthy contralateral carotid arteries or to stable, vulnerable carotid arteries (Figure 4B). The EC marker, CD31, was not detected in mouse carotid artery lesions, indicating a physical disruption of the endothelial layer induced both by the cuff placement for the injured arteries and the sectioning of the control arteries, as previously described. Double immunofluorescence examination of Atg16L1 and the macrophage marker F4/80 showed no colocalization in either hyperplastic or vulnerable carotid arteries (Figure 1 in the online-only Data Supplement). In sharp contrast, Atg16L1 and SMC-α-actin showed a strong colocalization in early atherosclerotic carotid plaques developing intimal hyperplasia 4 weeks after ligation (Figure 4B), but such colocalization was not observed in advanced atherosclerotic carotid plaques showing plaque vulnerability features (Figure 4B).

These data suggest that Atg16L1 expression might be induced at an early initial stage of the atherogenic process.
ATG16L1 Expression and Apoptosis Activation

Apoptosis through caspase-3 activation has recently been described as an important regulator of ATG16L1 stability or degradation in Crohn’s disease. Therefore, we analyzed whether caspase-3 cleavage could be related to ATG16L1 expression in human carotid atherosclerotic plaques. ATG16L1 expression area correlated positively with human carotid plaque contents of cleaved caspase-3 ($r=0.186$; $P=0.026$). ATG16L1 and cleaved caspase-3 showed a relative colocalization in the fibrous cap and the shoulder region of human carotid plaques as observed by double immunofluorescence examination (Figure 5A).

We further determined whether ATG16L1 stability, as well as autophagy flux, could be modulated during human macrophage foam cell formation and apoptosis. Human macrophages were differentiated from monocytes isolated from buffy coats of healthy donors and treated with oxLDL, an atherogenic stimulus known to induce lipid accumulation, inflammatory response, autophagy, and apoptosis (Figure 5B–5D).

As expected, a physiological dose of oxLDL (25 μM) increased LC3-II expression, the gold standard autophagy marker, in primary macrophages, in a time-dependent manner as compared with native-LDL. The autophagy flux also increased over time as assessed by LC3-II after pharmacological inhibition of autophagy by Bafilomycin A1, which prevents the fusion of the autophagosome to the lysosome (Figure 5B). Expression of ATG16L1 uncleaved and cleaved β isoforms also increased over time after exposure to physiological dose of oxLDL (Figure 5B). No modulation in caspase-3 expression and no detection of the cleaved caspase-3 isoform were observed by immunoblotting in the lysates of these human macrophages.

Macrophages treated with a cytotoxic oxLDL dose (100 μM) for 24 h showed significantly decreased ATG16L1 expression, as well as decreased autophagy flux compared with the cells treated with lower oxLDL doses (Figure 5C).

When the human primary macrophages were treated with a cytotoxic oxLDL dose (100 μM) during 48 h, the expression of cleaved caspase-3 was detected by immunofluorescence (Figure 5D). Colocalization between ATG16L1 and cleaved caspase-3 was not observed in primary macrophage foam cells.

Discussion

Recent studies have provided new insights into the importance of autophagy to maintain cardiovascular functions that are potentially relevant during atherogenesis. In this study, ATG16L1, an essential protein involved in the first steps of the autophagy process, was analyzed in carotid atherosclerotic plaques. This characterization of ATG16L1 protein expression in advanced human carotid atherosclerotic plaques and in mouse carotid plaques during the atherothrombotic process demonstrates that ATG16L1 is associated with characteristics of atherosclerotic lesion destabilization at early and advanced stages of atherosclerotic plaque progression.
These findings provide evidence that the essential autophagy ATG16L1 protein is abundantly expressed in advanced human atherosclerotic plaques in the necrotic core areas and the areas surrounding them, particularly in the shoulder regions. During the atherosclerotic process, retention of subendothelial lipoproteins attracts highly inflammatory cells, which engulf the lipid pools and secrete cytokines, chemokines, and proteolytic enzymes until they die, leaving behind cell debris and cholesterol crystals, forming a necrotic core. Over time, a complex feature of the atherosclerotic lesion in humans is characterized by a large necrotic core covered by a fibrous cap of variable thickness and shoulder regions with local infiltration of immune cells that actively produce inflammatory cytokines and enzymes. The presence of ATG16L1 in these active regions of human atherosclerotic lesions strongly suggests that this protein, which is involved in the early stage of autophagy, plays a significant role in atherosclerosis, at least in its late stages in humans.

Because autophagy is a ubiquitous catabolic process induced by a plethora of physiological and atherogenic stimuli, such as cholesterol crystals, hypoxia, reactive oxygen species, or oxLDL, ATG16L1 expression was anticipated to show a diffuse and homogenous distribution in advanced atherosclerotic plaques. Instead, high levels of ATG16L1 correlate with plaque areas rich in lipid and phagocytic cells, whereas poor in contractile SMCs, which is the classical phenotype of a vulnerable plaque. Although descriptive, to our knowledge, the present work is the first one to show ATG16L1 expression in human atherosclerotic plaques, thereby strengthening the link between autophagy pathways and an inflamed plaque phenotype.

Indeed, ATG16L1 expression was significantly positively correlated with proinflammatory cytokines, chemokines,
and MMPs in the plaque, suggesting a close connection between ATG16L1 and inflammatory pathways in atherosclerotic lesions. Among the 34 conserved Atg genes and their corresponding proteins involved in the molecular machinery of autophagy in mammals, ATG16L1 is the first autophagy marker which has been directly related to immunity and inflammation. Indeed, defective autophagy has repeatedly shown to lead to general immune disorders. In Crohn’s disease, a polymorphism in the ATG16L1 gene is associated with an excessive production of cytokines, such as interleukin-1β and interleukin-6 in peripheral blood mononuclear cells. Further, studies in macrophages from Atg16L1-deficient mice demonstrated that direct interaction between Nod2 and Atg16L1 is a key mechanism by which autophagy controls the endotoxin-induced inflammatory immune response through the translocation of procytokines into the lysosome. Additional investigations are needed to understand how ATG16L1 could regulate innate immunity in atherosclerosis.

In the present study, mast cells, highly potent immune cells, were sources of ATG16L1 in human carotid atherosclerotic lesions, as shown by colocalization with the mast cell–specific

Figure 5. ATG16L1 expression and apoptosis activation in human carotid atherosclerotic plaques and human primary macrophages. A, Double immunofluorescence labeling for ATG16L1 (red; Alexa-594-tagged) and cleaved caspase-3 (green; Alexa-488-tagged) in the fibrous cap area or in the shoulder area of carotid atherosclerotic plaque. Nuclei were stained with DAPI (blue) and Nomarski (gray). Scale bar=25 μm. Protein expression of ATG16L1, LC3, caspase-3, and β-actin in whole lysates of human primary macrophages treated with native-LDL (nLDL) or oxidized-LDL (OxLDL; 25 μg/mL) during different time points (B) or during 24 h (C) at different doses of OxLDL. The cells were pretreated with Bafilomycin A1 (Bafilo; 100 nM) during 2 h as indicated. The uncleaved and cleaved isoforms of ATG16L1 were detected through a short or long exposure time, respectively, of the immunoblots. D, Double immunofluorescence labeling for ATG16L1 (red; Alexa-594-tagged) and cleaved caspase-3 (green; Alexa-488-tagged) in human primary macrophages treated with oxLDL (100 μg/mL) during 48 h. Nomarski (gray). Scale bar=10 μm. L indicates lumen; and LDL, low-density lipoprotein.
protease tryptase. Importantly, in a 3-year follow up study, it was found that patients with high content of mast cells in endarterectomized carotid atherosclerotic plaques experienced significantly more cardiovascular events than the patients with lesser quantities of mast cells in the plaques.26 Indeed, it has repeatedly been observed that the number of mast cells in atherosclerotic plaques and their state of activation contribute to plaque vulnerability. On activation, mast cells produce and release granules which contain different mediators, such as heparin, histamine, proteases, growth factors, proinflammatory cytokines, and chemokines, including interleukin-6, TNF-α, monocyte chemoattractant protein-1, and regulated on activation T-cells expressed and secreted.27 Interestingly, in bone marrow–derived mast cells from Agt7-deficient mice, it has recently been demonstrated that autophagy plays a crucial role in the release of secretory granules from mast cells,28 thus potentially rendering autophagy of mast cells in advanced human atherosclerotic plaques a potential target for suppression of their plaque-weakening effects. However, further mechanistic investigations are needed to understand how ATG16L1 could affect mast cell functions during the atherogenic process.

We also provide evidence that phagocytic cells are important cellular sources of ATG16L1 in human carotid atherosclerotic lesions as observed by colocalization with CD68, a marker of macrophages in atherosclerotic lesions. A positive correlation between the areas of ATG16L1 staining and Oil Red O lipid staining suggests an expression of this autophagy protein in foam cells. Although autophagy has been involved in the clearance of macrophage lipid droplets and shown to contribute to cholesterol efflux from macrophage foam cells in mice, emerging data suggests that autophagy proteins could also be recruited into the phagosome, accelerating the degradation of cell corpses by the phagocytes through the noncanonical autophagy pathway named LC3-associated phagocytosis.11,29 The full molecular machinery involved in the cross-talk between autophagy and phagocytosis remains to be elucidated.

The immunogold labeling of ATG16L1 in foamy α-actin-positive SMCs demonstrates that SMCs are a cellular source of ATG16L1 in advanced atherosclerotic plaques. In agreement with this finding, recent data have shown a large contribution of intimal SMCs in foam cell formation in advanced atherosclerotic lesions where 50% of the CD68-positive cells expressed SMC markers.30 Further, studies on human primary aortic SMCs revealed that exposure of SMCs to oxidized LDL induced not only the expression of autophagy markers but also the expression of phagocytic markers.31 SMCs transition from a contractile to a migratory and synthetic phenotype, reflected by a typical loss of SMC markers, such as SMC-α-actin, myosin SMC heavy chains, and vimentin, has been reported in later stages of atherosclerosis32 and could be supported by the negative correlation between ATG16L1 and SMC-α-actin staining observed here. When considering this negative correlation with the fact that lipid-phagocytosing α-actin-positive SMC which are gaining CD68-positivity, that is, converted to macrophage foam cells, will retain their α-actin positivity,30 we can infer that our finding of a positive correlation between the areas of ATG16L1-positivity and the areas of Oil Red O positivity (neutral lipid content) and CD68-positive macrophages reflects rather the presence of ATG16L1 in monocyte-derived macrophages than in SMC-derived macrophages. Based on this characterization of ATG16L1 protein expression in human atherosclerotic plaques, we hypothesize that ATG16L1 might be involved in earlier stages of the atherogenic process during plaque development when macrophage foam cells are formed, and so ultimately contribute to destabilization of the plaque.

Atg16L1 expression was also analyzed in atherosclerosis-prone mice. For this purpose, we obtained carotid samples at different stages of plaque progression in a specific model of apolipoprotein-E-deficient mice, in which ultimately plaque vulnerability can be induced by incomplete ligation and cuff placement. Importantly, this validated and well-characterized mouse model of vulnerable atherosclerotic plaques induced by blood flow alteration display similar features as the human vulnerable carotid plaque, for example, a large lipid core, accumulation of macrophages in the shoulder region, a fibrous cap, the integrity of which can be disrupted with superimposed thrombosis.20,33 Interestingly, we observed that contractile SMCs expressing α-actin were cellular sources of Atg16L1 only in stable carotid atherosclerotic lesions developing intimal hyperplasia. Similar to the findings observed in the advanced human carotid atherosclerotic plaques, no colocalization between SMC-α-actin and Atg16L1 was observed in the carotid arteries of mice with vulnerable atherosclerotic lesions, suggesting that ATG16L1 might be involved in SMC function and survival particularly at an early stage of the atherosclerotic plaque progression.

Indeed, this study provides evidence of an increased ATG16L1 expression during macrophage foam cell formation, a crucial step in the initiation of atherosclerosis. At cytotoxic oxLDL levels, an impairment of autophagy flux was associated with a decrease in ATG16L1 expression in primary human macrophages. Furthermore, it has recently been demonstrated that ATG16L1 contains caspase cleavage motifs and that caspase-3 is directly involved in the degradation of the protein. Furthermore, a concomitant decrease in ATG16L1 stability and an increase in caspase-3 cleavage were observed in primary macrophages when treated with high doses of TNF-α and cycloheximide, an inhibitor of protein synthesis.18 In the present study, at a cellular level, our data suggest that apoptosis and caspase-3 activation might not be involved in the ATG16L1 degradation when foam cell formation is triggered by oxLDL.

At the tissue level, in the human carotid atherosclerotic plaque, the colocalization between ATG16L1 and cleaved caspase-3 indicate that other or a combination of stimuli might trigger ATG16L1 degradation by caspase-3. Of note, recent data have suggested that ischemic stress, such as moderate hypoxia, could potentiate inflammatory response in human primary macrophages.34 Therefore, further analysis of different atherogenic stimuli in different cell types will be needed to better describe the pathways involved in the regulation of ATG16L1 expression.
Finally, recent data from autophagy-deficient mice suggest that macrophage autophagy could be a potential therapeutic target for the treatment of atherosclerosis.\textsuperscript{19} In the present study of human carotid atherosclerotic plaques, we emphasize the need to better delineate the role that autophagy plays, besides the macrophages, in other cells of the vessel wall, such as ECs, SMCs, and mast cells, during the development of atherosclerosis.

Several limitations of the present study need to be considered. First, examination of atherosclerotic lesions in the longitudinal axial direction has demonstrated that upstream and downstream segments of the plaque exhibit different patterns of blood flow, significantly influencing the morphology and the composition of the lesion. In this study, only 1 mm fragment was used for histology because the rest of the material was used for quantitative biochemical analysis, so examination of these transversal sections only allows partial observations of ATG16L1 expression in human atherosclerotic plaques. To the best of our knowledge, the present investigation is the first to report endothelial cells as a major source of autophagy activity in human atherosclerotic plaques, but we did not investigate whether and how ATG16L1 could influence endothelial function during atherogenesis. Further studies will be needed to understand the putative link between ATG16L1, blood flow pattern, and endothelial dysfunction.

Furthermore, the mechanistic studies of ATG16L1 expression in macrophage foam cell formation were performed on macrophages differentiated from monocytes isolated from healthy individuals, which may not reflect the phenotype of resident macrophages present in human advanced atherosclerotic plaques. Further ex-vivo studies on cells extracted and isolated from the human atherosclerotic plaques will be needed to better understand the contribution of ATG16L1 in inflammatory responses in resident macrophages and other immune cells.

Taken together, these findings support a role of the autophagy protein ATG16L1 in atherogenesis and development of plaque vulnerability, thus suggesting a new member in this pathophysiological pathway.

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Disclosures

None.

References


Autophagy is a cell survival mechanism which can be upregulated in response to several atherogenic signals. Human genetic approaches have demonstrated that ATG16L1, an essential protein during early stages of the autophagy process, is involved in the pathogenesis of inflammatory disease, such as Crohn’s disease. However, it is still unknown whether ATG16L1 plays an important role in atherosclerosis development. This study provides the first evidence that the essential autophagy protein ATG16L1 is abundantly expressed in active regions of human carotid atherosclerotic plaques, particularly rich in lipids, phagocytic, and inflammatory cells, which corresponds to characteristics of vulnerable plaques. Interestingly, characterization of ATG16L1 expression during human primary macrophages foam cell formation and in carotid atherosclerotic plaques from a murine model of plaque vulnerability revealed that ATG16L1 is also involved in earlier stages of plaque formation. This study is pioneer in showing that ATG16L1 is linked to atherosclerotic plaque vulnerability.
ATG16L1 Expression in Carotid Atherosclerotic Plaques Is Associated With Plaque Vulnerability

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Materials and methods

Human atherosclerotic tissues
One-hundred and forty three human carotid atherosclerotic plaques from the Carotid Plaque Imaging Project (CPIP) were studied. This biobank includes carotid plaques from patients undergoing carotid endarterectomies at Skåne University Hospital, Malmö. The indications for surgery were plaques associated with ipsilateral symptoms (transient ischemic attack, stroke or amaurosis fugax) and stenosis higher than 70%, or plaques not associated with symptoms but with stenosis larger than 80%. Eighty-two of the patients had suffered symptoms. Informed consent was given by each patient. The study was approved by the local Ethics review board.

Assessment of cytokines, matrix metalloproteinases (MMPs) and cleaved caspase-3 was performed by ELISA in plaque homogenates as previously described. As controls, 5 human mammary arteries free of atherosclerosis were obtained from the Advanced Study of Aortic Pathology (ASAP). This biobank includes tissue biopsies from patients undergoing elective open heart surgery for aortic valve disease and/or ascending aortic disease as described previously.

Histology
After surgical removal, carotid plaques were snap-frozen, and fragments of 1 mm from the most stenotic region were obtained for histology. Transversal cryosections from the fragments were stained for plaque histology markers α-SMC-actin, CD68, Oil red O and Masson trichrome, as described previously. When staining for ATG16L1, primary antibody polyclonal rabbit anti-ATG16L1 (PM040, MBL International Corporation) and secondary antibody polyclonal goat anti-rabbit (DakoCytomation, Glostrup, Denmark) were used. Areas of the different stainings in the plaque (% area) were quantified blindly using Biopix iQ 2.1.8 (Gothenburg, Sweden) after scanning with ScanScope Console Version 8.2 (LRI imaging AB, Vista California, USA) and photographed with Aperio image scope v.8.0 (Aperio, Vista California, USA).

Immunoelectron microscopy
After surgical removal, another 1 mm fragment of the carotid plaque, consecutive to the one used for histology, in the most stenotic region was fixed in 3 % paraformaldehyde in 0.1 M phosphate buffer, washed and then infiltrated into 2.3 M of sucrose and frozen in liquid nitrogen. Ultrathin sectioning was performed at -95°C and placed on carbon-reinforced formvar-coated, 50 mesh nickel grids. Immunogold labelling procedure was performed as follows: grids were placed directly on drops of 2% BSA (Sigma fraction V) and 2% Fish gelatin (GE Healthcare, Buckinghamshire, UK) in phosphate buffer to block non-specific binding. Sections were then incubated with the rabbit anti-ATG16L1 antibody in phosphate buffer containing 0.1% BSA + 0.1% Gelatin over night in a humidified chamber at room temperature. The sections were thoroughly washed in the same buffer and bound antibodies were detected with protein A coated with 10 nm gold (Biocell, BBInternational, Cardiff, England). Sections were rinsed in buffer and fixed in 2% glutaraldehyde and contrasted with 0.05% uranyl acetate and embedded in 1% methylcellulose and examined in a Tecnai 10 (FEI company, Eindhoven, The
Netherlands) at 100 kV. Digital images were taken by a Veleta camera (Soft Imaging System GmbH, Münster, Germany).

Mouse model of atherosclerotic plaque vulnerability
Analyses of Atg16L1 expression were performed in an atherosclerotic carotid plaque rupture model in ApoE-deficient mice and contralateral control carotid arteries of the same mice. The model in brief consists of an incomplete ligation (Vicryl 5-0 suture, Ethicon Endo-Surgery Inc, Blue Ash, USA) of the common right carotid artery (just below the bifurcation) for 4 weeks, which triggers intimal hyperplasia and non-ruptured carotid atherosclerotic lesions. To provoke rupture of the developed plaque, a conical polyethylene cuff is placed proximal to the ligation site for 4 days. Approximately 50% of the 16 week old male mice display features of ruptured plaques, such as endothelial cracks or ulcers, and intraluminal thrombus formation. After sacrifice, injured and control carotid arteries are embedded (Cryomount, Histolab AB, Gothenburg, Sweden) and snap frozen. All experiments have been approved by the Stockholm Regional Board for Experimental Animal Ethics.

Immunofluorescence
To identify in which cell types the autophagy marker ATG16L1 is expressed in human and mouse atherosclerotic plaques, we used fluorescent double-staining. Acetone-fixed cryosections were first incubated with rabbit anti-ATG16L1 antibody (PM040, MBL International Corporation) at 4°C overnight, followed with AlexaFluor 594 labeled goat anti-rabbit IgG (Life technologies, Stockholm, Sweden) for 1h. Subsequently, the sections were incubated overnight with mouse anti-CD68 antibody (Clone KP1, DAKO, Stockholm, Sweden), mouse anti-SMC-α-actin antibody (Clone1A4, DAKO, Stockholm, Sweden), mouse anti-CD31 antibody (Clone JC70A, DAKO, Stockholm, Sweden) or mouse anti-human mast cell tryptase antibody (Clone AA1, DAKO, Stockholm, Sweden) followed by AlexaFluor 488 labeled goat anti-mouse IgG (Life technologies, Stockholm, Sweden), with subsequent staining of the nuclei using DAPI. To better identify macrophages in the mice carotid atherosclerotic plaques, sections were incubated with rat anti-F4/80 (Clone BM8, Abcam, Stockholm, Sweden) followed with AlexaFluor488 labeled goat anti-rat IgG (Life technologies, Stockholm, Sweden). For the fluorescent double staining between cleaved caspase-3 and ATG16L1, the sections were incubated with mouse anti-ATG16L1 antibody (M150-5, MBL International Corporation, Woburn, USA) then with rabbit anti-cleaved caspase-3 antibody (9961, Cell signaling, Danvers, USA). The specificity of the ATG16L1 antibody was confirmed by incubation with isotype-matched control IgG. Images were obtained using a Zeiss LSM700 confocal laser microscope using x20, 0.8NA objective lens (for the atherosclerotic lesions) or using a Leica SP5 confocal laser microscope using x100 oil, 1.4NA objective lens, (for the macrophages). Each image consisted of a Z-stack of 15 to 20 optical slices taken at 0.3μm intervals.

Primary human macrophage studies
To determine whether oxidized-LDL could stimulate ATG16L1 expression, human monocytes were isolated from fresh buffy coats of healthy donors (Blood Transfusion Center, Karolinska University Hospital, Stockholm, Sweden) as previously described. In brief, human peripheral blood mononuclear cells were isolated from buffy coats by endotoxin-free Ficoll density gradient centrifugation. Monocytes were then separated from lymphocytes by high density hyper-osmotic Percoll density gradient centrifugation and separated from platelets and dead cells on a low-density iso-osmotic Percoll density gradient. Monocytes were cultured in RPMI-1640 medium (Invitrogen, Stockholm, Sweden) supplemented with penicillin-streptomycin, L-glutamine (2mM) and 5% FBS (Invitrogen, Stockholm, Sweden). The cells were seeded in 6-well plates at a density of 0.8×10^6 cells/mL and differentiated in the presence of recombinant mouse M-CSF (100 ng/ml; PeproTech, Stockholm, Sweden) over seven days. The primary human macrophages were then treated with home-made copper-oxidized LDL (OxLDL) at different time points (2, 6, 24 or 48 hours) or at different dose (25, 50 or 100μg/mL). Native-LDL (nLDL, 25μg/mL) was used for controls.

For the autophagy flux assessment, the cells were pretreated during 2h with Bafilomycin A1 (100nM; B1793, Sigma-Aldrich, Stockholm, Sweden). Protein expression of ATG16L1, LC3, Caspase-3 and β-Actin were analyzed in the whole cell lysate by immunoblotting using rabbit anti-ATG16L1 antibody (PM040, MBL International Corporation, Woburn, USA), rabbit anti-LC3 antibody (NB100-2220, Novus biological, Cambridge, UK), anti-rabbit caspase-3 antibody (9962, Cell signaling, Danvers, USA) and anti-mouse β-actin antibody (Sigma-Aldrich, Stockholm, Sweden) as previously described.6

Statistics
Variables are presented as percentages or mean (standard deviation, SD), median (inter-quartile range, IQR), depending on their distribution (normal or not, respectively). Spearman rho was used to determine correlations. Significance was considered at \( P<0.05 \). SPSS 20.0 was used for the statistical analysis.

References


Supplemental figure I. Detection of Atg16L1 expression in frozen sections of carotid artery from a model of vulnerable carotid atherosclerotic plaque development in Apolipoprotein E-deficient mice. Representative cross-sections of the control left carotid artery without injury, stable plaque in the right carotid artery after 4 weeks of incomplete ligation + 4 days of cuff placement, and vulnerable plaque in right carotid artery after 4 weeks + 4 days ligation/cuff treatment. Double immunofluorescence labeling for Atg16L1 (red, Alexa-594-tagged) and F4/80 macrophage marker (green, Alexa-488-tagged). Nuclei were stained with DAPI (blue) and Nomarski indicates differential interface contrast microscopy (grey). Scale bar 50µm. L: lumen.