An Imbalance Between CD36 and ABCA1 Protein Expression Favors Lipid Accumulation in Stroke-Prone Ulcerated Carotid Plaques

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Background—CD36 is a macrophage scavenger receptor mediating the uptake of modified lipoproteins, whereas the ABCA1 transporter counteracts this effect by mediating cellular lipid efflux. Based on a DNA microarray, we previously found that the CD36 and ABCA1 genes were overexpressed in symptom-causing carotid plaques (CP) compared with nonsymptom-causing CP. To evaluate their role in CP destabilization, we conducted detailed immunohistochemical studies on the localization of lipids, CD36 and ABCA1 proteins, extravasated red blood cells, and atheromatous/necrotic tissue.

Methods—Ninety-two high-grade (>70%) stenosing CP obtained from carotid endarterectomy were Oil-red-O–stained for evaluation of neutral lipids. Subgroups of nonsymptom-causing and symptom-causing CP (n=42) were further analyzed by immunostaining adjacent histological sections against CD36 and ABCA1 and examining them microscopically.

Results—When compared with nonsymptom-causing CP, the amount of extracellular lipid and the expression of CD36 protein were elevated in symptom-causing CP, but no difference was found in ABCA1 expression. These observations were also confirmed when ulcerated and nonulcerated CP were compared. In ulcerated CP, CD36 protein expression was higher than that of ABCA1, and the opposite was true in nonulcerated CP. CD36 colocalized with extravasated red blood cells and atheromatous or necrotic areas in the various types of CP.

Conclusions—Our results suggest that an imbalance between lipid influx (CD36) and efflux (ABCA1) favors lipid accumulation in macrophages of ulcerated CP, thus contributing to plaque destabilization. Furthermore, colocalization of CD36 protein with red blood cells suggests that intraplaque hemorrhages may contribute to the lipid load and thus the stability of CP. (Stroke. 2010;41:389-393.)

Key Words: foam cell formation ■ immunohistochemistry ■ lipid transport

Advanced atherosclerotic plaques in the internal carotid artery wall are considered as a clinical threat potentially leading to stroke. A spectrum of factors underlying the final destabilization and rupture of the plaques has been studied, but distinguishing between high-risk and low-risk plaques is still an enigma. However, one of the morphological features of high-risk plaques seems to be a vast lipid-filled core, the formation of which is fueled by the death of engorged lipid-filled macrophage foam cells.

In a genome-wide microarray expression study of advanced carotid plaques (CP), we discovered that the transformation of a CP from nonsymptom-causing to symptom-causing coincided with increased expression of macrophage adipophilin,4 a lipid-loading marker. We also found that CD36 and ABCA1 genes were overexpressed in symptom-causing CP, and the result was replicated by real-time reverse-transcription polymerase chain reaction for CD36 (Saksi et al, unpublished data, 2009). Interestingly, both CD36 and ABCA1 seem to play an important role in balancing macrophage lipid intake and efflux. Oxidized low-density lipoprotein-scavenging CD36, a multifunctional membrane glycoprotein, is considered essential for foam cell formation,1 and its expression is further stimulated by its own ligand.2 This vicious cycle potentially leading to fatal cholesterol overload is partly inhibited by ABCA1, a membrane transporter protein of ATP-binding cassette (ABC) family, widely supported in its atheroprotectiveness, removing free cholesterol and phospholipids, and necessary for the initiation of macrophage reverse cholesterol transport.3

On the basis of the differential expression of these 2 proteins in our DNA microarray, we wanted to also study immunohistochemically the expression of CD36 and ABCA1
proteins and their relation to the presence and localization of lipids and lipid-filled foam cells, plaque ulceration, extracellular red blood cells (RBC), and atheromatous/necrotic areas in single CP. We also related the data to previous investigations of the same Helsinki Carotid Endarterectomy Study (HeCES) cohort.4–8

Materials and Methods

Ninety-two patients underwent carotid endarterectomy because of high-grade (70%–99%) carotid artery stenosis in digital subtraction angiography according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria between years 1997 and 2000. Operable carotid stenoses with a diameter reduction of 70% to 99% in digital subtraction angiography according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET) were included. Symptom status was based on clinical criteria. Stroke, TIA, or amaurosis fugax within 120 days before CEA was considered a culprit symptom. Patients with other potential sources of cerebral embolism were excluded. From all the specimens, a subgroup (n=14) of completely asymptomatic CP and another subgroup (n=23) of stroke-causing CP were selected for immunohistochemical analysis on CD36 and ABCA1. The macroscopic characteristics of the CEA specimens and the presence or absence of plaque ulceration were recorded in situ by an experienced vascular surgeon. Blood samples were collected before the CEA, and each patient underwent a neurological investigation, a recording of medical history, and cerebral imaging with MRI or CT. Before surgery, all the enrolled patients gave signed informed consent, and the study was approved by the appropriate Ethics Committees.

Figure 1. Ninety-two patients underwent carotid endarterectomy (CEA) because of high-grade (70%–99%) carotid artery stenosis between the years 1997 and 2000. Operable carotid stenoses with a diameter reduction of 70% to 99% in digital subtraction angiography according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET) were included. Symptom status was based on clinical criteria. Stroke, TIA, or amaurosis fugax within 120 days before CEA were considered a culprit symptom. Patients with other potential sources of cerebral embolism were excluded. From all the specimens, a subgroup (n=14) of completely asymptomatic CP and another subgroup (n=23) of stroke-causing CP were selected for immunohistochemical analysis on CD36 and ABCA1. The macroscopic characteristics of the CEA specimens and the presence or absence of plaque ulceration were recorded in situ by an experienced vascular surgeon. Blood samples were collected before the CEA, and each patient underwent a neurological investigation, a recording of medical history, and cerebral imaging with MRI or CT. Before surgery, all the enrolled patients gave signed informed consent, and the study was approved by the appropriate Ethics Committees.

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Relative CD36 and ABCA1 gene expression was determined using the comparative CT method and standard curve method, respectively, and expression of β-actin was used for normalization.

Because of the small sample size, we used nonparametric statistical tests. Ordinal variables were tested with Mann–Whitney U and Jonckheere–Terpstra tests, and continuous variables were tested with Spearman rank correlation. To adjust for confounding factors such as age, gender, body mass index, blood low-density lipoprotein concentration, and the amount of plaque macrophages, a multinomial logistic regression model was used to explore the association between regions of interest levels and ABCA1/CD36, as described in detail previously.4 Statistical analyses were performed using SPSS (version 14.0).

Results

The relative amount of CD36 mRNA correlated with mean CD36 protein expression within plaques (r_s=0.551; P<0.001), with the latter being observed in a subgroup of macrophage foam cell-formed rims around the lipid cores (Figure 3A). CD36 mRNA and protein levels were higher in ulcerated than in nonulcerated CP (P=0.014; P=0.001; Figure 2A). The mRNA and protein expressions of adipophilin and CD36 were correlated (r_s=0.370, P=0.024 and r_s=0.422, P=0.022, respectively). Within each region of interest, CD36 expression associated with the presence of RBC and atheromatous/necrotic areas (P<0.001 and P=0.006; Figure 2C).

ABCA1 expression was mostly co-distributed with macrophages (Figure 3C). The relative expressions of ABCA1 mRNA and protein were in good correlation (r_s=0.367; P=0.05), but neither of them differed when ulceration (Figure 2B) or symptom status was compared. ABCA1 mRNA and protein correlated with increased CP adipophilin mRNA and protein expressions (r_s=0.419, P=0.030 and r_s=0.404, P=0.030). In CP with
an increased amount of intracellular lipids, the expression of ABCA1 protein was elevated ($P=0.005$).

The regional balance of CD36 and ABCA1 protein expressions was analyzed in separate regions of interest categorized into 3 groups according to their ratio. In ulcerated CP, there were significantly more regions of interest with higher CD36 than ABCA1 expression, whereas the opposite was true for nonulcerated CP ($P=0.006$; Figure 3D). ABCA1 expression was increased when compared with CD36 expression in patients with higher-plasma high-density lipoprotein cholesterol concentrations ($P=0.001$). Moreover, the amount of adipophilin mRNA was significantly decreased in CP with higher ABCA1 than CD36 expression ($P<0.001$).

Extracellular lipid deposits were significantly increased in symptom-causing and ulcerated CP ($P=0.040$ and $P=0.038$, respectively). Intraplaque hemorrhages were associated with a higher amount of extracellular CP lipid ($P=0.002$). The intracellular lipid load associated with both Fas-receptor and Fas-ligand expression ($P=0.042$; $P=0.041$). Adipophilin protein expression was increased in plaques with more total lipid deposits ($P=0.031$).

Neither the traditional cardiovascular risk factors nor medications had important associations to plaque CD36 or ABCA1 expression or CP lipid status. For more detailed analysis on immunohistochemical results, please see Figure I.

Discussion

Uptake of modified low-density lipoprotein particles by multiple mechanisms with ensuing foam cell formation is an essential part of the atherosclerotic process.$^1$ We found that the amount of extracellular lipid was elevated in unstable CP, and that a shifted balance in the expression of 2 key lipid trafficking proteins, CD36 and ABCA1, may favor progressive lipid accumulation into macrophages and lead to a stroke-prone, ulcerated CP.

In the CP of the HeCES study, induction of $CD36$ and $ABCA1$ genes was initially identified by DNA microarray studies. These results showed a marked $>2.1$-fold increase in $CD36$ gene expression and also suggested a 1.5-fold increase in $ABCA1$ gene expression in symptomatic CP in comparison with asymptomatic ones (Saksi et al, unpublished data, 2009), whereas the corresponding fold changes were 1.1 ($CD36$) and 1.6 ($ABCA1$) for ulcerated and nonulcerated plaques. Added to this, the present immunohistochemical analysis revealed novel data that potentially help us better understand the clinical significance of CD36 and ABCA1.

In line with the mRNA results, we found increased CD36 protein expression in ulcerated CP compared with nonulcerated CP (Figure 2A). This difference was also seen between symptom-causing and nonsymptom-causing CP. We also found that CD36-expressing macrophages colocalized with extravasated RBC (Figure 2C), and that the extracellular lipid load of CP associated with the presence of intraplaque hemorrhages. Intraplaque hemorrhages and cholesterol-containing RBC membranes are assumed to represent one potential source of lipids, fueling the formation of foam cells,$^4,9$ probably mediated through CD36.

Despite the mRNA results, no significant difference in ABCA1 protein expression was found between the CP subgroups (Figure 2B). Discordance between relative ABCA1 mRNA and protein expression levels in human CP and in murine tissues has been reported previously.$^{10}$ Interestingly, it has been proposed that when sufficient amounts of ABC transporters are expressed, macrophages are able to handle excess of lipids,$^2$ supporting the notion that the formation of atheromatous lesions is largely a failure of lipid efflux mechanisms, ie, ABCA1. The cleaning of harmful oxidized low-density lipoprotein with the aid of scavenger receptor-mediated lipid intake is beneficial, but not feedback-regulated.$^{11}$ Thus, a failure in lipid efflux and subsequent cytotoxic intracellular loading of free cholesterol could lead to the demise of macrophage foam cells. Probably as a preventive mechanism, it has been found that intracellular lipid loading through oxidized low-density lipoprotein uptake induces the biosynthesis of both ABCA1 and CD36 proteins, partially through the same oxidized low-density lipoprotein-induced PPAR$\gamma$-LXR$\alpha$/RXR pathway that couples lipid intake and efflux.$^2$

However, our protein level data indicated differential regulation of $CD36$ and $ABCA1$ translation, because CD36 mRNA and protein expression was clearly elevated in ulcerated CP, whereas protein expression of ABCA1 was not. Moreover, the CD36/ABCA1 ratio appears as one potential

Figure 2. CD36 protein expression was increased in ulcerated CP when compared with nonulcerated CP (A). However, such a difference was not seen with ABCA1 protein expression (B). CD36 protein expression associated with the presence of extravasated RBC (C) ($\pm$1 SE).
factor associated with and potentially explaining the cardinal CP features. There was higher CD36 than ABCA1 expression in ulcerated CP, whereas the contrary was true for nonulcerated CP, indicating that the relative imbalance in the expression levels of these proteins favors lipid influx and retention in ulcerated CP (Figure 3D). Also, the expression of adipophilin mRNA, a marker of cellular lipid loading, was elevated in CP with higher CD36/ABCA1 ratio; this ratio also elevated with the degree of carotid stenosis.

The discordance between ABCA1 mRNA and protein expressions may underlie the elevated CD36/ABCA1 ratio. Multiple intracellular and extracellular causes may affect ABCA1 expression and stability.10 However, the potentially hazardous CD36/ABCA1 imbalance could also be partly explained by excessive CD36 expression. We have shown CD36 overexpression in stroke-prone CP in line with recent data showing higher serum levels of soluble CD36 in symptomatic carotid artery disease patients.12 One mechanism here could be mediated through high-density lipoprotein, which may have a regulatory feedback role that could diminish CD36 mRNA expression13 because, in our material, elevated high-density lipoprotein levels, which are usually considered atheroprotective, associated with a lower CD36/ABCA1 ratio. However, the precise composition of the plaque microenvironment and its effect on macrophage lipid transporters are still unclear.

**Conclusion**

Although the present data do not establish the causes underlying the observed unfavorable balance between the 2 investigated opposing lipid transport molecules, they remain an interesting subject for further research. In conclusion, our present results indicate that ABCA1 protein appears to be insufficiently expressed in relation to the increased plaque lipid burden. Macrophages may locally be unable to express sufficient amounts of ABCA1 to prevent fatal intracellular lipid accumulation. The death of foam cells turns intracellular lipids into extracellular lipids, contributing to enlargement of the lipid-rich acellular atheroma,14 and appearing as CP with larger diameter. Interestingly, in our CP material, intracellular lipid-loading was associated with Fas-receptor and ligand expression, which is considered an important signaling pathway of apoptosis in atherosclerosis.15

As more and more macrophages are recruited around the core areas rich in cell debris and free lipids, a vicious cycle is fueled, leading to further growth of the atheroma and eventual plaque destabilization and ulceration. This would explain the elevated amount of extracellular lipid found in the ulcerated plaques and the plaques from symptomatic patients, indicating that enhanced turnover of foam cells is a general feature in clinically significant stenosing carotid artery disease. Even though knockout studies have shown that prevention of foam cell formation is beneficial, the solution for therapeutic strategies may not lie in decreasing scavenger receptor expression, but rather in developing pharmacological agents that increase ABCA1 expression or prevent its degradation.

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Disclosure
None.

References
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