Adipophilin Expression Is Increased in Symptomatic Carotid Atherosclerosis
Correlation With Red Blood Cells and Cholesterol Crystals

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Background and Purpose—Adipophilin is an adipose differentiation–related protein expressed in lipid-containing cells. Using DNA microarray analysis, we previously found the adipophilin gene (ADFP) to be overexpressed in symptomatic carotid plaques (CP). This led us to further examine the role of adipophilin in carotid atherosclerosis relative to symptom status.

Methods—Ninety-eight high-grade (>70%) CPs were obtained in carotid endarterectomy. The relative expression of ADFP mRNA was measured by quantitative real-time RT-PCR, and the relative amount of adipophilin protein was quantified with Western blotting. Detailed topographical correlations with extravasated red blood cells and extracellular cholesterol crystals were obtained by means of immunohistochemistry.

Results—The relative expression of ADFP mRNA was increased in symptomatic compared with asymptomatic CPs at both the mRNA level (1.82±0.19(SE) versus 1.25±0.15, P=0.012) and the protein level (1.04±0.23 versus 0.46±0.14, P=0.043). Adipophilin colocalized with macrophage foam cells, extravasated red blood cells (P<0.0001), and cholesterol crystals (P<0.0001), and its expression associated with macroscopic ulceration of CP (P<0.0001).

Conclusions—Intraplaque hemorrhages may contribute to intracellular lipid accumulation and consequent adipophilin expression. Because adipophilin blocks cholesterol efflux from lipid-laden cells, they may die and develop a necrotic lipid core, thereby destabilizing the plaque. (Stroke. 2007;38:1791-1798.)

Key Words: adipophilin ■ atherosclerosis ■ carotid artery disease ■ gene expression ■ immunohistochemistry

The role of lipids is well established in the pathophysiology of atherosclerosis.1–3 The permeability of dysfucntional atherosclerotic endothelium for blood lipoproteins increases, and blood-borne inflammatory cells accumulate within the vessel wall. The majority of lipoprotein particles captured into the arterial wall becomes modified, which leads to their uptake by macrophages and subsequent macrophage transformation into foam cells.4

Based on our previous DNA microarray analysis, we concluded that ADFP (the adipophilin gene) belongs to the set of genes induced selectively in symptomatic carotid plaques (CP) compared with asymptomatic CPs showing 1.7-fold induction in symptomatic plaques.5 Adipophilin, an adipose differentiation–related protein, is found in lipid-containing cells,6 including macrophage foam cells. Its expression in macrophages is enhanced by modified LDL,7 and when expressed, it further enhances lipid accumulation and prevents lipid efflux from lipid-laden macrophages.8 Because the expression of adipophilin seems to be elevated in atherosclerotic lesions compared with healthy arterial intima, it may play a role in the pathogenesis of atherosclerosis, notably in the transformation of macrophages into foam cells.7

To confirm our finding of increased expression in symptomatic CPs by microarray analysis, we measured the relative expression of ADFP mRNA by quantitative real-time RT-PCR and also quantified the relative expression of adipophilin protein in a larger set of symptomatic (SCPs) and asymptomatic carotid plaques (ACPs). Finally, to describe adipophilin expression in symptom-generating carotid lesions and its correlation with atherogenic cells and substances residing within the plaque, we studied CPs morphologically by scoring the immunohistochemical findings with detailed

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topographical correlations with red blood cells (RBCs) and cholesterol crystals.

**Materials and Methods**

**Subjects and Samples**

Details of patient selection, baseline characteristics (Table), and the carotid endarterectomy procedure of Helsinki Carotid Endarterectomy Study (HeCES) have been described previously. Study patients (n=93) were collected consecutively during the years 1995 to 2000, and they underwent digital subtraction angiography, which revealed 1 high-grade (>70%) stenosis in the internal carotid artery according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria. The patients were classified as either symptomatic or asymptomatic based on their medical history, and they were considered to have large artery atherosclerosis according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria. Symptomatic patients had experienced either a transient ischemic attack, stroke, or amaurosis fugax. Of all patients, the vast majority of the plaques belonged to the subgroup VIabc, ie, they were ulcerated plaques with a luminal thrombus and an intraplaque hemorrhage. The specimens were cut into 5 longitudinal slices, each containing the segment of the tightest stenosis of the internal carotid artery plaque. Blood samples were taken before the carotid endarterectomy for blood lipid analysis, and cerebral CT or MRI scans were taken from all patients. All patients gave written informed consent. The study was approved by the Ethics Committees of the Departments of Neurology and Surgery of Helsinki University Central Hospital.

**RNA Extraction and Quantitative Real-Time RT-PCR for ADFP mRNA**

Total cellular RNA was extracted with Trizol reagent (Invitrogen Life Technologies) and purified with the RNeasy Total RNA Isolation Kit (Qiagen) according to the manufacturer’s recommendations. Quantitative real-time RT-PCR was performed using Assays-on-Demand Gene Expression Products and ABI PRISM 7000 Sequence Detection System (Applied Biosystems) according to the manufacturer’s recommendations. Relative ADFP gene expres-
expression was determined by the comparative Ct method, normalizing expression to \( \beta \)-actin and GADPH.

**Protein Isolation and Western Blotting**

Total cellular proteins were isolated from the phenol-chloroform phase left over from the RNA extraction with Trizol reagent. Protein samples were quantified by the Bradford method, and 15 \( \mu \)g was separated on 10% SDS-polyacrylamide gel electrophoresis followed by electroblotting on to PVDF membranes (Hybond P, Amersham Biosciences UK Limited). Successful transfer of proteins was confirmed using Ponceau S staining. Membranes were blocked in 5% skimmed milk and 0.1% Tween 100 in tris-buffered saline. Adipophilin (American Research Products) and \( \beta \)-actin (Sigma-Aldrich) were detected using mouse monoclonal antibodies at working dilutions of 1:500 and 1:4800, respectively. Peroxidase-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA) was used as secondary antibody. Proteins were visualized using ECL Plus Western Blotting Detection Reagents and Typhoon 9400 Variable ImageQuant TL 1D Gel Analysis v2003.1 software all according to the manufacturer’s recommendations (Amersham Biosciences). The relative amount of adipophilin protein is given as a ratio of the total fluorescence signal intensities of adipophilin and \( \beta \)-actin, which results in semiquantitative ratios of proteins in the sample.

**Immunohistochemistry and Light Microscopy**

The specimens were fixed in Carnoy’s fluid and embedded in paraffin. The primary antibodies used were mouse monoclonal antibodies against adipophilin (same as in Western blotting; working dilution 1:100), against CD34 (Novocastra Laboratories Ltd; working dilution 1:200), and against CD68 (Dako; working dilution 1:200). Mouse IgG1 (Dako, Glostrup, Denmark) and IgG3 (ID Labs Inc., Glasgow, UK) were used in equivalent dilutions as a negative control. Because 11 CPs were excluded attributable to extensive calcification, the total number of CPs available for immunohistochemistry was 87. For each antibody, sections were stained immunohistochemically as previously described, except that the sections were deparaffinized with xylene, rinsed in distilled water for 2 minutes, embedded in citrate buffer, and warmed in a microwave oven at 800W for 2 \( \times \)5 minutes and at 600W for 5 minutes. Citrate buffer and distilled water were added before each step to prevent the specimens from drying. The specimens were cooled down at room temperature and washed with PBS. To remove endogenous peroxidase, the sections were embedded in 0.3% solution of hydrogen peroxidase and methanol, followed by 2 \( \times \)5 minutes washes with PBS. After this, the samples were incubated in a humidified chamber for 30 minutes in 10% normal horse serum (Vector laboratories, Inc, Burlingame, CA, USA) to block nonspecific staining.

Double stainings were performed essentially as described above, but after the first primary antibody application the sections were treated with the Novolink polymer detection system (Novocastra Laboratories Ltd) according to the manufacturer’s instructions, and the chromogen DAB (3,3’-diaminobenzidine) was applied after washing with PBS. The sections were then boiled 4 minutes in the microwave oven (750W) in 1mMol EDTA (pH 8), after which the second primary antibody was applied. Further detection was carried out with Novolink followed by DAB +Ni as the chromogen, without counter staining (Hematoxylin).

Light microscopy (Axioplan 2, Carl Zeiss) was performed by 2 investigators (K.N. and P.M.I.) blinded to the clinical data. To obtain a detailed distribution data from immunohistochemical stainings, we applied a method adapted from a previous approach by Kockx and coworkers. All immunohistochemically stained sections were photographed with a digital camera (AxioCam MRC, Axioplan 2), using the smallest objective (1.25x). The individual images were pieced together with the imaging software, and a grid layer was positioned onto each image. Thus, for further evaluation, we obtained an image of the whole specimen compartmentalized by a squared grid (Figure 1), which consisted of 2 \( \times \)2 mm ROIs (region of interest) that were further evaluated for specific morphological parameters, and compared with different histopathological regions of plaque activity.

Immunohistochemically detected expression of adipophilin in each ROI was graded semiquantitatively into 4 groups: 0=no staining in the ROI, 1=stained area (A) one-third or less of the ROI, 2=stained area one-third <A \( \leq \)two-thirds of the ROI, and 3=more than two-thirds of the ROI stained positive for adipophilin. The presence of RBCs and cholesterol crystals was evaluated as follows: 0=not present in the ROI and 1=present in the ROI. A subgroup (n=7) of morphologically complicated plaques were stained with anti-CD34 (a marker for endothelial cells) to evaluate the presence of microvessels, and graded semiquantitatively from 0 to 3 (0=no vessels present in the ROI, 1=moderate number of vessels, 3=many vessels). The individually graded immunoreactivities were used to calculate the mean adipophilin protein immunoreactivity.

**Statistical Analysis**

Statistical analyses of baseline characteristics were performed using parametric (t test and Pearson correlation; continuous normal variables), nonparametric (Kruskall-Wallis and Mann–Whitney U test; non-normal variables) or exact (Fisher exact test; binary variables) statistical tests. These were performed by means of SPSS for Windows software (version 10.07, SPSS).

To explore the associations between covariates (cholesterol crystals, red blood cells and ulceration) and ordinal outcome (adipophilin in ROIs) we performed statistical analysis using fixed effects...
proportional odds regression model. Each covariate was analyzed univariately (no other risk factors) and multivariately (blood cholesterol concentration, BMI, age and gender). Additionally, to take into account the grid based multiple measurements of adipophilin ROI levels in each specimen, where the grid of ROIs cover all the area of a specimen, we fitted also mixed effects proportional odds model. Because results of the mixed effects model did not change conclusions, we report results from the fixed effects model. All reported significance levels (probability values) for studied variables are from the multivariate model. Technical details and results of the statistical analysis are presented in the supplement, available online at http://stroke.ahajournals.org.

Results

Overexpression of ADFP mRNA in Symptomatic CPs

The relative expression of ADFP mRNA was analyzed in the whole material, and it was significantly higher in stroke-causing CPs than in ACPs, revealing 1.5-fold induction (1.82±0.19 versus 1.25±0.15, Mann–Whitney U: P=0.012, Figure 2A). There was also more ADFP mRNA expression in the CPs that contained macroscopic ulceration (Mann–Whitney U: P<0.001), and ADFP mRNA expression was also associated with the degree of CP stenosis (Spearman correlation: r=0.328, P=0.002). It further associated with the amount of CD163 mRNA (r=0.677, P<0.001) and HO-1 mRNA (r=0.751, P<0.001), as assessed previously. Men had clearly more intense ADFP mRNA expression (Mann–Whitney U: P=0.035), patients with diabetes (Mann–Whitney U: P=0.005) or hypertension (Mann–Whitney U: P=0.022) had more ADFP mRNA, but no other risk factors of atherosclerosis (eg, age, smoking, BMI, coronary disease, peripheral artery disease, dyslipidemia, medication, or time delay between symptom and surgery) had any associations with ADFP mRNA expression.

Quantification of Adipophilin Protein Expression by Western Blotting

Adipophilin protein was quantified in relation to β-actin by Western blotting from the extremes subgroup (n=22). The expression of adipophilin protein relative to β-actin was significantly higher in SCPs than ACPs (1.04±0.23 versus 0.46±0.14, t test: P=0.043, Figure 2B and 2C). Adipophilin protein levels correlated with the ADFP mRNA levels (r=0.480, P=0.024). Men had significantly more adipophilin (t test: P=0.0003), but no other risk factors of atherosclerosis (eg, age, smoking, BMI, diabetes, hypertension, coronary disease, peripheral artery disease, dyslipidemia, medication, or time delay between symptom and surgery) had any associations with adipophilin protein expression.

Immunohistochemistry and Colocalization Analysis

When examined morphologically, adipophilin was often present near the atheromatous/necrotic regions of a plaque (Figure 3A), especially around the lipid core, as well as in the proximity of extravasated RBCs (Figure 3B) and cholesterol crystals (Figure 3A and 3C). Clusters of adipophilin-positive cells were also seen within the healthy portions of the vessel wall and underneath the endothelium (Figure 3D), but not particularly in the shoulder regions. Double stainings revealed that most of the adipophilin positivity localized within macrophages (Figure 3E and 3F).

Microscopic studies of each ROI of the plaque revealed striking colocalization of adipophilin protein and extravasated RBCs (Proportional odds model: P<0.0001; Figure 4A) and cholesterol crystals (Proportional odds model: P<0.0001; Figure 4B). Adipophilin expression was markedly increased in the CPs that had macroscopic ulceration compared with the CPs without ulceration (Proportional odds model: P<0.0001; Figure 4C).
model: P<0.0001). Microvessel analysis of the subgroup of plaques with the most multiform morphology revealed a tendency toward larger numbers of microvessels in ruptured and SCPs and a positive association between the presence of microvessels and extravasated RBCs (Mann–Whitney U: P=0.029).

The mean adipophilin protein immunoreactivity of each CP associated positively with the total plasma cholesterol concentration (Pearson correlation: \( r=0.222, P=0.045 \)), and there was a similar trend for the plasma LDL cholesterol concentration (\( r=0.234, P=0.061 \)). The mean adipophilin protein expression also associated with the macrophage count (\( r=0.379, P=0.001 \)), and with the amount of CD163 mRNA (\( r=0.215, P=0.048 \)) in these CPs as determined previously.5,10 In addition, it associated positively with the degree of CP stenosis (\( r=0.269, P=0.014 \)) and negatively with the presence of endothelium, as found previously9 (\( r=-0.306, P=0.005 \)). Men had more adipophilin immunoreactivity (t test: \( P=0.037 \)), and as expected, the mean adipophilin protein expression markedly correlated with ADFP mRNA expression (\( r=0.409, P=0.0002 \)). No other risk factors of atherosclerosis, medication, comorbidity, or time delay between symptom and surgery associated with adipophilin protein expression.

**Discussion**

DNA microarray results revealing differential expression of the ADFP in SCPs and ACPs5 led us to conduct a more detailed morphological study of the expression of adipophilin in carotid atherosclerosis and to examine its correlation with the CP phenotype, ie, symptomatic versus asymptomatic. First, we measured the relative expression of ADFP mRNA and found it is to be significantly increased in stroke-causing CPs compared with ACPs (\( P=0.012 \), Figure 2A). Secondly, we determined the amount of adipophilin protein and found it to be overexpressed in SCPs (\( P=0.043 \), Figure 2B). Thirdly, we elucidated the plaque morphology relative to adipophilin expression and found an association between adipophilin expression, extravasated RBCs, cholesterol crystals (Figure 4, \( P<0.0001 \)), and macroscopic ulceration of CP (\( P<0.0001 \)). Importantly, these latter findings link ADFP to the hallmarks of complicated atherosclerotic plaques, ie, those with atherothrombosis, and thus well agree with the observation of overexpression of adipophilin in the symptomatic CP phenotype.
A hallmark for advanced atherosclerotic lesions is a necrotic lipid core, which results from the balance between lipid inflow into LDL particles and lipid outflow into HDL particles. Lipoproteins, particularly LDL, can diffuse directly from blood into the arterial intima and then form lipid deposits. Although local modification of LDL particles appears to be the major driving force in the generation of cholesterol imbalance and ensuing accumulation within the plaque, intraplaque hemorrhages also appear to be a source of cholesterol, because extravasated erythrocytes can carry cholesterol in their membranes into the plaque. The eventual death of lipid-laden foam cells leads to further accumulation of extracellular lipids and contributes to the formation of a necrotic lipid core, and our data support a role for adipophilin in lipid accumulation.

Adipophilin protein is expressed in a wide range of tissues. It has been revealed that the cellular amount of adipophilin reflects the mass of neutral lipids stored within cells. Adipophilin has also been studied previously in atherosclerotic lesions. Accordingly, Larigauderie et al observed 3.5 times more intense adipophilin expression in atherosclerotic lesions than in normal intima and found most of the ADFP mRNA expression of atherosclerotic lesions to be present within lipid-rich macrophages. They suggested that adipophilin expression is a consequence of cholesteryl-ester retention within cells, and that adipophilin further increases cholesterol accumulation by inhibiting its efflux from cells. Wang et al also observed ADFP mRNA in macrophage-rich areas in atherosclerotic plaques, and they suggested that adipophilin may play a key role in the lipid metabolism of foam cells and hence ultimately contributes to the formation of a lipid core in human atherosclerotic lesions. We found that both ADFP mRNA and adipophilin protein expression are increased in symptomatic compared with asymptomatic carotid atherosclerosis. To our knowledge, this is the first time that adipophilin expression, at either the mRNA or the protein level, has been studied in atherosclerosis in clinical patients with different symptom status. It could be hypothesized that, by preventing lipid efflux from foam cells, adipophilin contributes to their death and thereby participates in the growth of the confluent necrotic lipid core and the instability of the plaque.

Similar speculations have been made by Faber and coworkers on perilipin, a protein very similar to adipophilin. They studied ruptured and unruptured human atherosclerotic plaques and found that perilipin mRNA and protein are overexpressed in ruptured compared with stable plaques. Perilipin is also present on intracellular triglyceride droplets of adipocytes and has been thought to be required for maximal lipolytic activity. Faber and coworkers suggested that the overexpression of perilipin in ruptured plaques might indicate reduced lipolysis of cholesteryl esters in those plaques, leading to increased lipid retention and plaque destabilization. These observations agree well with our findings and the interpretation that these lipolysis-regulating molecules may upset the balance of lipid traffic, leading to progressive lipid accumulation within an atherosclerotic plaque and eventually increase its vulnerability to rupture.

Adipophilin was expressed mainly in macrophages, especially in foam cells, and the most striking observation in this study was that adipophilin localized mainly to the areas where extravasated RBCs and cholesterol crystals were also found. Because erythrocyte membranes contain cholesterol in quantity and excess cholesterol can create a nidus for nucleation into crystalline cholesterol, this observation is in accordance with the idea that the presence of cholesterol crystals reflects previous intraplaque hemorrhages in that area. In addition, adipophilin expression was strongly increased in plaques that had macroscopic ulceration, which lends further support to potential role of intraplaque hemorrhages. The observation that microvessel density associated with the presence of extravasated RBCs suggests that small neovessels with weak endothelium are an important source for intraplaque hemorrhages. Therefore, intraplaque hemorrhages may have a bigger role in the lipid metabolism of an atherosclerotic lesion than previously assumed, as suggested by Virmani. Furthermore, the subsequent release of cholesterol from erythrocyte membranes and the ensuing oxidative

Figure 4. Adipophilin expression was associated with the presence of cholesterol crystals (A, Proportional odds method: $P<0.0001$) and extravasated RBCs (B, Proportional odds method: $P<0.0001$) in each ROI of an individual plaque.
and inflammatory stress could be important triggers in the induction of adipophilin expression within SCPs.

In this study, we have used all available plaque tissue for adipophilin mRNA and relative protein quantification. An alternative would be to use only tissue from carefully characterized areas of plaque morphology. Similarly, we and several others have adopted microarray analysis to the whole plaque, but some groups have used only specific areas of plaque activity in their analysis. Interestingly though, the results shared considerable similarity despite the different approaches used, eg, 30% of the gene expression changes we reported were also identified by Papaspyridonos and colleagues. This suggests that both approaches yield meaningful information and can be used to complement each other. However, there are some grounds in support of using whole plaque tissue. The selection of only specific plaque areas based on a priori hypotheses can be problematic. Conventional morphologic studies have been studied quite extensively in symptomatic and asymptomatic plaques, but very little rational morphology has been studied quite extensively in symptomatic and asymptomatic plaques. The selection of only specific plaque areas can be problematic. Conventional morphologic studies have been studied quite extensively in symptomatic and asymptomatic plaques, but very little rational morphology has been studied quite extensively in symptomatic and asymptomatic plaques.

In conclusion, our observation of increased ADFP mRNA and adipophilin protein expression in SCPs as well as the marked colocalization of adipophilin protein expression with extravasated RBCs and cholesterol crystals may suggest that intraplaque hemorrhages play a role in the vulnerability of symptomatic carotid plaques by contributing to lipid accumulation and inducing adipophilin expression, thereby further increasing lipid accumulation by preventing lipid efflux. The eventual death of lipid-laden macrophages would then accelerate the formation of a necrotic lipid core, which again renders the plaque unstable and so predisposes it to symptom generation. The mechanisms underlying this observation warrant further research, which will hopefully reveal new molecular targets for therapeutic applications stabilizing atherosclerotic plaques and preventing ischemic thromboembolic strokes.

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None.

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