

Overexpression of Transferrin Receptor and Ferritin Related to Clinical Symptoms and Destabilization of Human Carotid Plaques

WEI LI,^{*,1} LI-HUA XU,^{†,1,2} CLAES FORSSELL,[‡] JEROME L. SULLIVAN,[§] AND XI-MING YUAN^{†,3}

**Division of Pharmacology; †Division of Experimental Pathology, Department of Clinical and Experimental Medicine; ‡Division of Vascular Surgery, Department of Medicine and Health Sciences, Faculty of Health Sciences, Linköping University, Linköping, SE-58185 Sweden; and §Burnett College of Biomedical Sciences, University of Central Florida, Orlando, Florida 32816*

Accumulation of tissue iron has been implicated in development of atherosclerotic lesions mainly because of increased iron-catalyzed oxidative injury. However, it remains unknown whether cellular iron import and storage in human atheroma are related to human atheroma development. We found that transferrin receptor 1 (TfR1), a major iron importer, is highly expressed in foamy macrophages and some smooth muscle cells in intimal lesions of human carotid atheroma, mainly in cytoplasmic accumulation patterns. In 52 human carotid atherosclerotic lesions, TfR1 expression was positively correlated with macrophage infiltration, ectopic lysosomal cathepsin L, and ferritin expression. Highly expressed TfR1 and ferritin in CD68-positive macrophages were significantly associated with development and severity of human carotid plaques, smoking, and patient's symptoms. The findings suggest that pathologic macrophage iron metabolism may contribute to vulnerability of human atheroma, established risk factors, and their clinical symptoms. The cytoplasmic overexpression of TfR1 may be the result of lysosomal dysfunction and ectopic accumulation of lysosomal cathepsin L caused by atheroma-relevant lipids in atherogenesis. *Exp Biol Med* 233:818–826, 2008

Key words: atherosclerosis; apoptosis; iron metabolism; lysosomes; macrophages; plaque rupture

Introduction

Atherosclerosis is a chronic inflammatory process in which inflammation, immune activation, and oxidative stress are crucially involved. Some epidemiologic and many experimental studies suggest that development of atherosclerosis may be associated with the amount of iron storage in the human body (1, 2), which may lead to iron-driven oxidative stress and signaling in atherogenesis (3).

The transferrin receptor (TfR) is an essential protein involved in iron uptake and the regulation of cell growth. There are at least two types of TfR, TfR1 and TfR2. TfR1 expresses in many cell types, including macrophages and endothelial cells. Iron uptake occurs *via* the internalization of iron-loaded transferrin (Fe-Tf) mediated by the interaction with the TfR1. In addition, the TfR may also contain other growth-regulating properties. In contrast to substantial data on serum ferritin or transferrin in atherogenesis, very few studies on TfR expression and atherosclerosis have been done. Moreover, it is important to know whether ferritin accumulation in atherosclerotic lesions is associated with TfR expression in different types of atherosclerotic plaques.

Ferritin is an iron-storage and stress-related protein. Increased levels of iron and ferritin have been found mainly in CD68-positive macrophages of human atherosclerotic lesions and in the circulation of patients with atherosclerosis (2, 4, 5). Several studies have investigated pathological implications of increased ferritin expression in atherosclerotic lesions (4–6), in which lesion-dependent expression of ferritin is associated with pro-inflammatory cytokines and atheroma cell apoptosis (6). Macrophages and macrophage

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¹ These authors contributed equally to the work.

² Current address: Department of Cardiology, The Affiliated Hospital of Beihua University, Jilin City 132011, China.

³ To whom correspondence should be addressed at Division of Experimental Pathology, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden. E-mail: yuan.ximing@lnr.liu.se; yuanximing@gmail.com

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foam cells with increased expression of ferritin and high levels of iron may enhance oxidative stress, plaque vulnerability and cell death, as suggested by animal and pathological studies (7–10). A role for intralysosomal iron in promoting plaque progression is strongly supported by animal studies that show decreased lesion size and/or increased lesion stability after systemic *in vivo* iron depletion by chelation or dietary iron restriction (8–10).

There is substantial data that both ferritin and TfR1 can be degraded or internalized in lysosomes or endosomes (11, 12). In different types of cells, release of iron from ferritin occurs mainly through lysosomal proteolysis (12). The importance of lysosomes in iron-catalyzed low-density lipoprotein (LDL) oxidation in atherogenesis has been highlighted recently, although how lysosomes affect iron metabolism in human atherosclerotic lesions is still unknown (13).

Recent studies suggest that factors other than the degree of atherosclerotic stenosis are involved in ischemic pathogenesis. Rupture of vulnerable lesions is the main cause of both acute cardiac events and brain stroke. It has been demonstrated that both the mechanisms and the imaging modalities of plaque instability in the carotid circulation may well reflect those in the coronary circulation (14, 15). Human carotid atheromata from endarterectomy, therefore, presents a good model for identifying contributing factors in vulnerable plaque formation.

In the present investigation, we studied the expression and distribution of TfR1 and its relation to ferritin accumulation and lysosomal cathepsin L expression in human carotid atherosclerotic plaques, and then explored potential connections between their expressions, plaque development, and clinical severity.

Materials and Methods

Linköping Carotid Study and Human Carotid Atheroma. The Linköping Carotid Study is a prospective clinical-pathology study, which collects the atherosclerotic carotid arteries from patients who undergo carotid endarterectomy at Linköping University Hospital, Linköping, Sweden. The ethics committees of Linköping University Hospital approved the Linköping Carotid Study.

Fifty-two atherosclerotic carotid samples obtained from consecutive patients were included in the present study. A detailed clinical history of each patient was taken with particular reference to carotid territory ischemic events. The timing of the most recent symptom was used for dividing patients into groups for comparison. Patients without neurologic symptoms within 6 months from the plaque in question were designated as asymptomatic (Asym). Patients with hemispheric neurologic deficits lasting less than 24 hrs were designated as transitory ischemic attacks (TIA) and patients with nondisabling hemispheric neurologic deficits lasting more than 24 hrs were designated as minor stroke (MS).

Carotid endarterectomy arteries were from 52 patients (32 men and 20 women). The age range of patients was from 51 to 85 years (mean: 71.8 ± 1.1). Forty-four patients with cerebrovascular symptoms were classified as symptomatic and eight patients with no cerebrovascular symptoms were defined as asymptomatic. The degree of stenosis of carotid arteries was determined by examinations of carotid duplex ultrasound and computer tomography. In the symptomatic group, the average time from symptom onset to surgery was 51.3 days (range: 9–218 days). Several stroke risk factors were recorded, including hypertension (defined by hypertension history and diastolic blood pressure ≥ 110 mm Hg; $n = 39$), smoking (defined as regular smoking >5 years; $n = 19$), and diabetes mellitus (regular diabetes medication; $n = 9$). Lipid examination included total cholesterol, LDL cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides. All patients underwent pre-operative carotid artery duplex ultrasound scanning and had $\geq 50\%$ stenosis.

Tissue Processing. Carotid artery samples were collected immediately after endarterectomy. All operations were performed with minimal manipulation of the specimen and without opening the arterial lumen. The samples were divided into two parts; one part was directly frozen at -70°C and the second part was prepared for histopathology analysis after decalcification. Three to five transverse segments were taken from each specimen and fixed in 4% (w/v) formaldehyde and embedded in paraffin. These segments were sectioned with an interval of 2 μm .

Immunohistochemistry. After deparaffinization in xylene and rehydration in graded ethanols, serial paraffin sections were exposed to the following primary antibodies: monoclonal mouse anti-human CD68 (clone PG-M1; 1:100; DAKO, Glostrup, Denmark), mouse anti-human smooth muscle actin (clone 1A4; 1:200; DAKO), swine anti-human cathepsin L (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-human ferritin (1:200; DAKO), mouse monoclonal anti-human TfR1 (1:330; Alpha Diagnostic International, San Antonio, TX). The immunoreactions were visualized using the DAKO EnVision™+/HRP method or DAKO ChemMate™ EnVision™ Detection Kit (DAKO). Controls without the primary antibody were run for each protocol, resulting in consistently negative observations. Isotype controls were tested with normal serum from the same animal or species that provided the primary antibodies or the same immunoglobulin isotype. They were prepared using the same method used for the primary antibodies, and used with same diluent and working dilution. Sections were then counterstained with Gill's hematoxylin solution.

Collagen Staining. Collagen and smooth muscle cells were stained according to the Van Gieson method. The deparaffinized and hydrated sections were stained with 3% (w/v) hematoxylin for 10 mins and then with Van Gieson's solution (1% acid fuchsin and saturated picric acid) for 1

min. This method stains collagen a pink/red color, and stains smooth muscle cells yellow.

Classification of the Plaques. To examine whether the expression of TfR1 or ferritin is related to the development of plaques, all the plaques were classified into three groups based on their morphology and collagen staining. Some samples were excluded due to uncertainty of their plaque type. Type 1 plaque ($n = 8$): intact plaque, a fibrous cap $>100\ \mu\text{m}$, no lipid pool formation, no leukocyte infiltration and intact internal elastica membrane (IEM). Type 2 plaque ($n = 9$): intact plaque, lipid pool formation, infiltration of leukocytes and a fibrous cap $<100\ \mu\text{m}$. Type 3 plaque ($n = 19$): ruptured plaque, often containing large necrotic core, cholesterol crystals, infiltration of leukocytes, internal plaque hemorrhage (IPH), thrombosis, and neovascularisation.

Image Analysis. All the histologic sections were examined by a light microscope (Leits DMRBE Leica; Wetzlar, Germany) connected to a C2400 microscope camera (Hamamatsu; Photonics Norden AB, Solna, Sweden). The images were digitized to a Macintosh computer using Image Grabber software (version 1.2; Tucows Inc., Toronto, Ontario). The microscope was set on the same parameters for all sections using the same immunostaining at $\times 20$ magnification.

Eight fields in intima and seven fields in media were randomly selected for each section. The digitized images were analyzed with Adobe Photoshop (version 5.5; Adobe Systems Inc., San Jose, CA). The image analysis was done as described by Lehr *et al.* (16). We measured immunopositive area pixel values and immunonegative area pixel values (background area pixel values) for each image; the sum of the two was the total pixel area. Positive-stained areas for each section were presented as immunopositive areas (%), which were calculated as the average immunostained area per pixel value divided by the total pixel value.

All the histologic assessments were made blind to plaque classification and clinical characteristics by two observers.

Statistical Analysis. Continuous data were expressed as mean $\pm$ SEM. Differences between two groups were analyzed by the Mann-Whitney U test. Differences among three groups were compared by the Kruskal-Wallis test. Nominal data were examined using the chi-squared test. Correlations between immunohistochemical positive areas of TfR1, ferritin, CD68, and cathepsin L were examined by using the Spearman correlation test and presented as the Spearman correlation coefficient (r_s). All the P values were two-tailed and a value of $P < 0.05$ was considered statistically significant.

Results

TfR1 Massively Expressed in Both Macrophages and Smooth Muscle Cells in Intimal Lesions of Human Carotid Atheroma. Immunohisto-

chemical experiments showed that TfR1 was positively expressed in all the lesions from all the patients, which occurred mainly in the intima and macrophage-positive areas and some positive-stained areas of smooth muscle cells (Fig. 1A). TfR1-positive staining appeared in both cell membranes and cytoplasm (Fig. 1B); however, cytoplasmic accumulation was the dominant pattern of positive TfR1 staining. Some endothelial cells were also positive to TfR1. Quantitative immunohistochemical analysis showed that both TfR1 and ferritin were enriched in atheroma intima, in contrast, to a less degree, in the media ($P < 0.0001$ for both comparisons) (Fig. 1C).

Quantitative immunohistochemical analysis also demonstrated that positive-stained areas and intensities of TfR1 were significantly correlated with CD68-positive macrophages (see below) but not with smooth muscle cells, though the serial sections expression of TfR1 (Fig. 1D) was seen in many CD68-positive cells and some smooth muscle cells (Fig. 1E).

TfR1 Expression Is Positively Correlated with Macrophage Infiltration, Lysosomal Cathepsin L, and Ferritin Expression. We next investigated the correlation of TfR1 expression with iron-storage protein ferritin, lysosomal cathepsin L, and macrophage infiltration on serial-sectioned carotid atherosclerotic lesions. We found that TfR1 (Fig. 2A) and ferritin (Fig. 2B) positivities were often located in the same CD68-positive areas in atherosclerotic carotid lesions (Fig. 1D and E). Moreover, TfR1 immunopositive areas were partially colocalized with lysosomal cathepsin L that predominantly accumulated in the nuclear region of many foamy cells (Fig. 2C). The Spearman correlation test was used to examine possible correlations between the expressions of the above immunomarkers. As shown in Figure 2D and E, TfR1 immunopositive areas in these lesions significantly correlated with those of ferritin-positive and CD68-positive areas. More interestingly, TfR1 immunopositive areas were significantly correlated with the expression of cathepsin L (Fig. 2F), and cathepsin L but not TfR1 significantly correlated to apoptosis (data not shown here). The expression of TfR1 was not significantly correlated with smooth muscle cells ($n = 51$, $r_s = -0.168$, $P = 0.24$). There was a significant correlation between immunopositive areas of ferritin-positive and CD68-positive macrophages ($n = 51$, $r_s = 0.62$, $P < 0.0001$). These data together suggested that iron-transport protein TfR1 is associated with iron accumulation and ectopic lysosomal cathepsin expressions in human atherosclerotic lesions.

Highly Expressed TfR1 and Ferritin in CD68-Positive Macrophages Are Associated with Instability and Rupture of Human Carotid Plaques. All the plaques included in the study were divided into three groups according to their collagen contents and morphologic alterations. The expression of TfR1 in different types of human carotid atherosclerotic lesions was then further explored. Compared to stable lesions (Fig. 3A; type 1

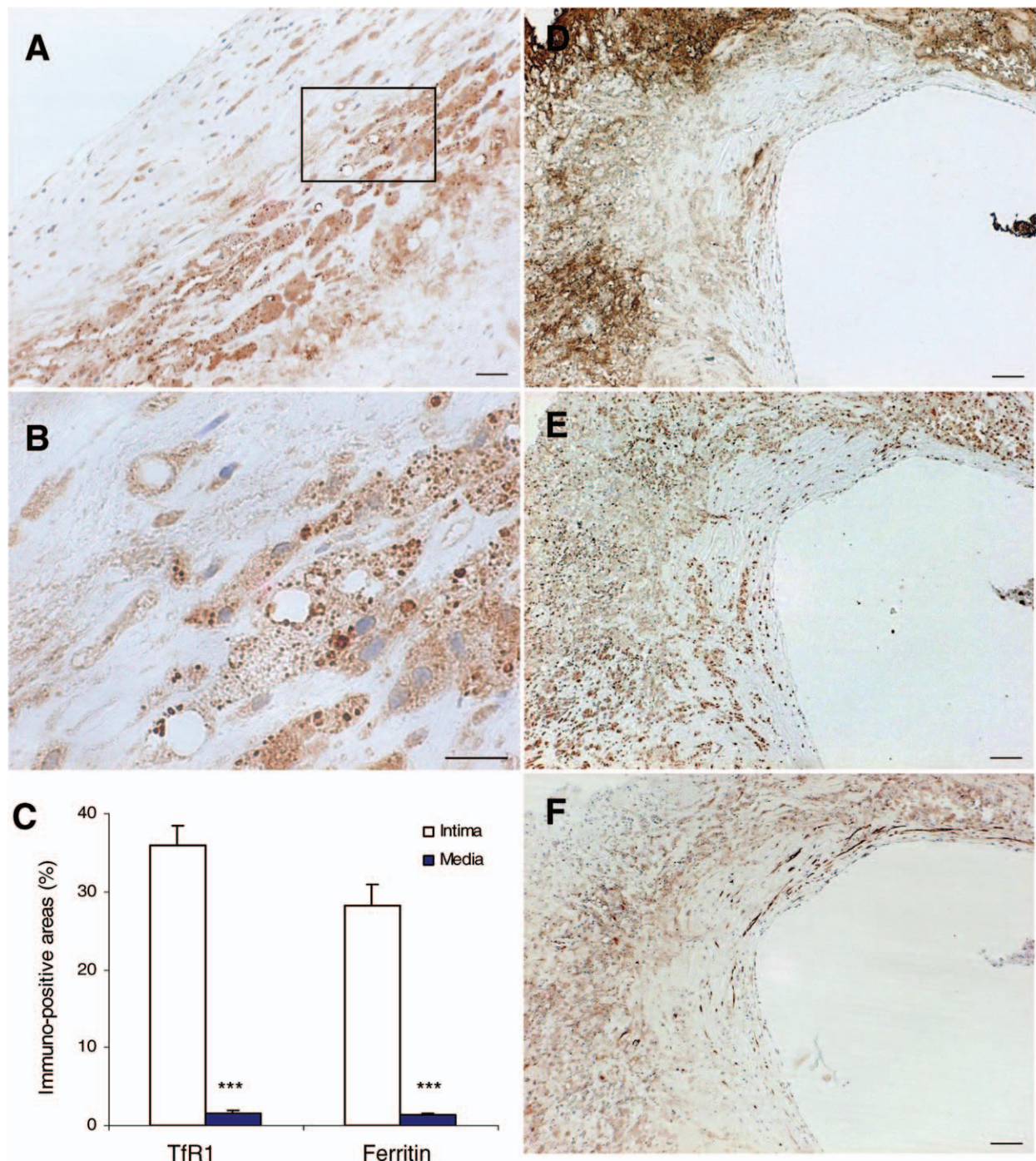


Figure 1. TfR1 expressed in both macrophages and smooth muscle cells in human carotid atherosclerotic lesions. (A) Positive TfR1 immunostaining was seen in intimal region of human carotid atheroma. (B) A higher-power of the area in the black box of A showed that TfR1 had both cytoplasm and plasma membrane expression. (C) Quantitative analysis of TfR1 or ferritin expression in intima and media ($n = 52$). Immunohistochemistry of TfR1 (D), CD68 (E), and smooth muscle actin (F) on serial sections showed that CD68-positive macrophages and actin-positive smooth muscle cells were both enriched in TfR1. Bar in A = 40 μm , B = 20 μm , and D–F = 100 μm . *** $P < 0.0001$ versus immunopositive area in intima areas. A color version of this figure is available in the online journal.

plaque), TfR1 expression is remarkably increased in unstable lesions (Fig. 3B; type 2 plaque) and ruptured lesions (Fig. 3C; type 3 plaque). Stronger staining was present around necrotic core regions (Fig. 3B) and ruptured areas in the lesions (Fig. 3C). As shown in Figure 3D, E, and F, the immunopositive areas of TfR1, ferritin, and

CD68 were increased in type 2 and type 3 plaques. TfR1 immunopositive areas in both type 2 and type 3 plaques were significantly greater in comparison with type 1 plaques. The immunopositive areas of CD68 and ferritin were also significantly greater in type 3 than type 1 plaques.

In addition, the proportion of asymptomatic and

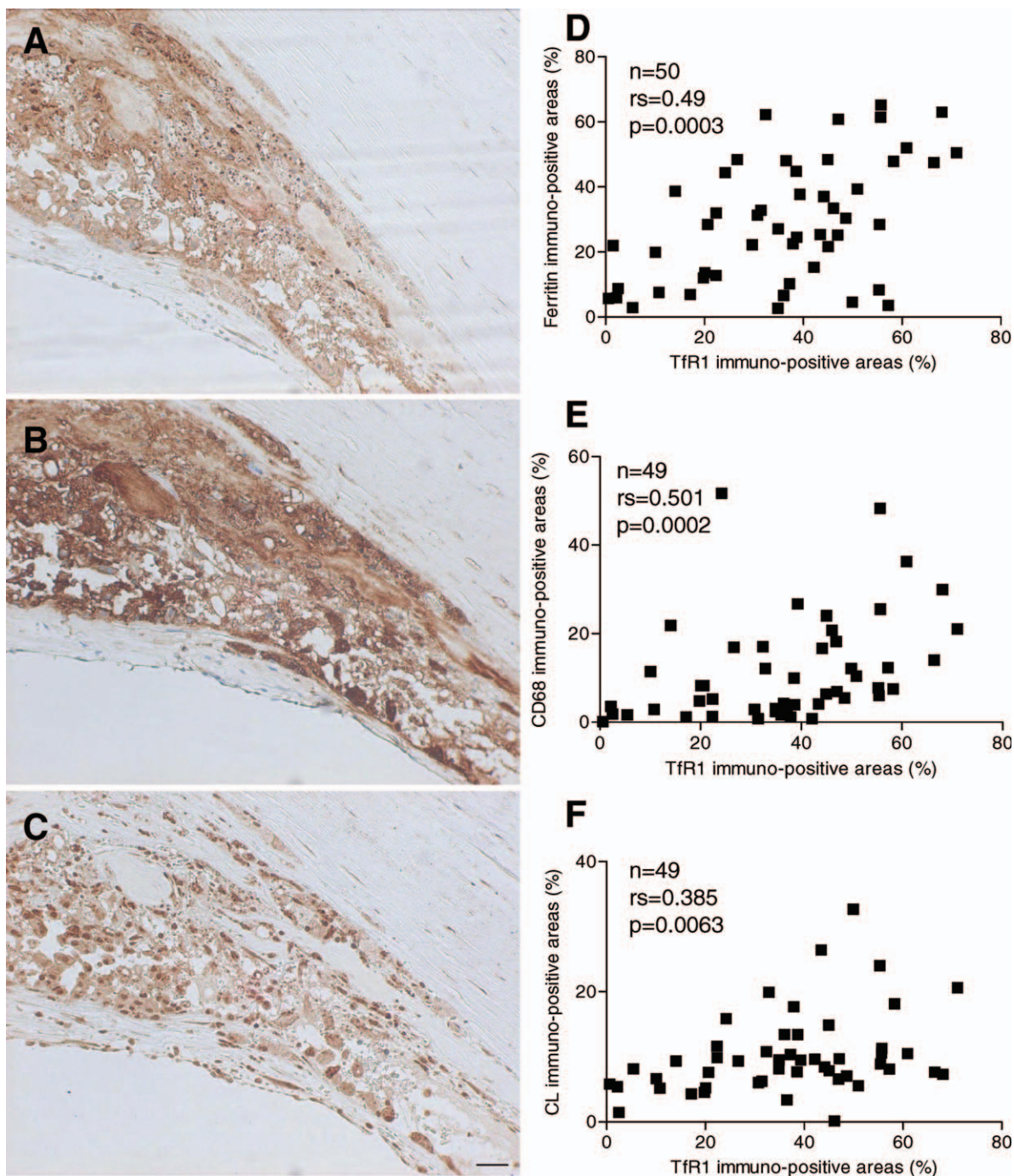


Figure 2. Tfr1 expression in macrophages was significantly correlated to the expression of ferritin and lysosomal cathepsin L (CL). On serial sections of a human carotid atheroma, the positive staining of Tfr1 (A), ferritin (B), and cathepsin L (C) appeared in the same region. Quantitative analysis of the immunostained sections showed that Tfr1 was significantly correlated with ferritin (D), CD68-positive macrophages (E), and lysosomal cathepsin L (F). Bars = 40 μ m. A color version of this figure is available in the online journal.

symptomatic cases was 3:5 in type 1 plaques (37.5%), 0:9 in type 2 plaques (0%), and 1:18 in type 3 plaques (5.3%). There were more asymptomatic cases in the type 1 plaque group than those in the type 2 and type 3 groups ($P < 0.05$).

Expression Levels of Tfr1, Ferritin, and CD68-Positive Macrophages Were Significantly Higher in Plaques of Symptomatic Patients. To explore the

association between the above markers and clinical symptoms of carotid atheroma, we examined possible differences in immune-positive areas of Tfr1, ferritin, and CD68 between symptomatic and asymptomatic patients. Significant differences were found in expression levels of Tfr1 and CD68 between two groups of patients, which suggested that macrophage Tfr1 might be related to clinical

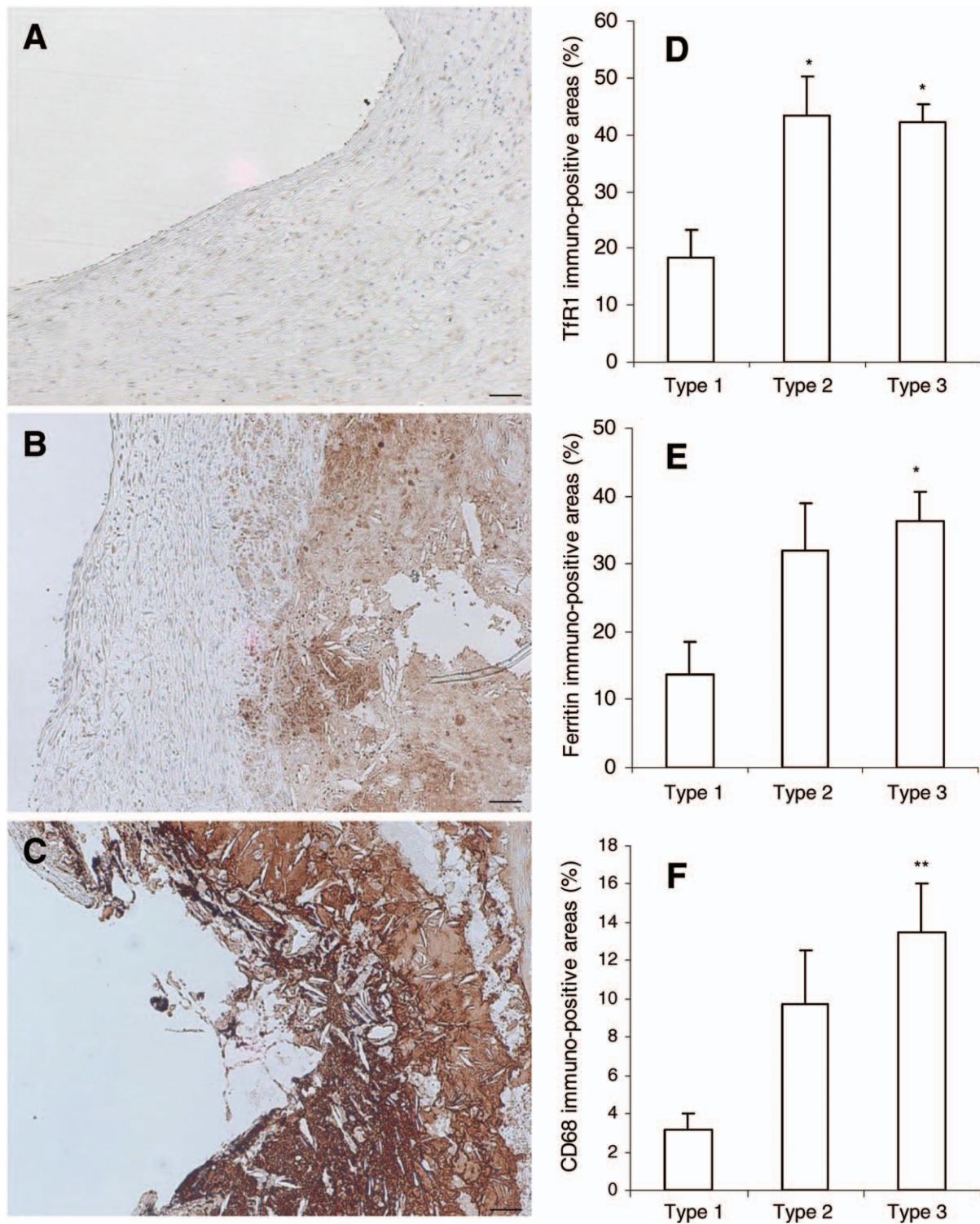


Figure 3. Both Tfr1 and ferritin in macrophages were significantly related to development of human carotid atherosclerotic plaques. (A–C) Localization of Tfr1 expression in type 1 (A), type 2 (B), and type 3 (C) lesions. (D–F) Quantitative immunohistochemical analysis of Tfr1 (D), ferritin (E), and CD68 (F) showed that compared to type 1 plaques ($n = 8$), type 2 ($n = 9$), and type 3 ($n = 19$) plaques contain higher levels of Tfr1, ferritin, and macrophages. * $P < 0.05$, and ** $P < 0.01$ versus type 1 plaques (Bars = 100 μm). A color version of this figure is available in the online journal.

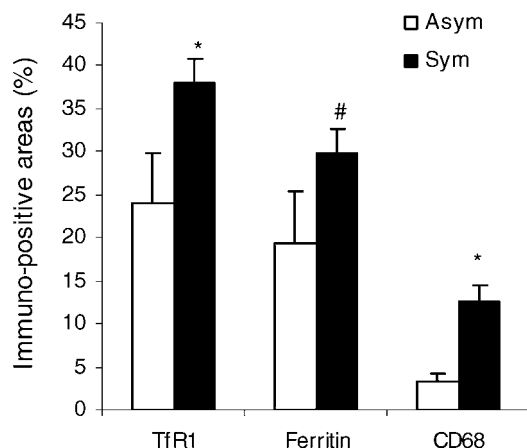


Figure 4. The carotid atheroma from symptomatic patients contained significantly higher levels of TfR1, ferritin, and CD68. Quantitative analysis of immunopositive areas showed that patients with clinical symptoms (Sym; $n = 43$ for TfR1, ferritin, and CD68) had significantly higher levels of TfR1 and CD68-positive macrophages in their lesions compared to asymptomatic patients (Asym; $n = 8$ for TfR1 and CD68, $n = 9$ for ferritin). * $P < 0.05$ and # $P = 0.1$ versus Asym.

symptoms of carotid atheroma (Fig. 4). Ferritin-immunopositive areas were also greater in the symptomatic group as compared to asymptomatic group, but the difference was not significant (29.9 ± 2.78 vs. 19.4 ± 6.07 ; $P = 0.1$). Because of the variation in days from symptom onset until surgery in this study (range: 9–218 days), we further examined the potential effects of length of time before surgery on significant alterations described above in different type of plaque groups and symptom groups. There was no statistical difference among these groups.

Smoking but Not Hypertension Was Significantly Associated with TfR1 Expression in Human Carotid Atheroma. To assess possible interactions between some of the well-established risk factors and iron metabolism, the immunopositive areas of TfR1 expression were compared between patients with or without diabetes mellitus, hypertension, or smoking habit (Fig. 5). Smoking had a significantly stronger influence upon TfR1 expression (Fig. 5A; $P = 0.04$). The differences were still significant when patients with diabetes were excluded (Fig. 5B; $P = 0.03$), which suggests that increased expression of TfR1 may be one additional mechanism for smoking as a risk factor of atherosclerosis progression. The greater levels of TfR1 expression were also seen in patients with diabetes mellitus compared with patients without diabetes mellitus (Fig. 5A). This difference was clearer and nearly statistically significant when patients with a smoking habit were excluded from diabetes group (Fig. 5B; $P = 0.053$). Hypertension had no apparent influence on TfR1 expression. Among examined factors, HDL cholesterol was the only recorded risk factor that was significantly different between asymptomatic vs. symptomatic patients (1.9 ± 0.4 vs. 1.1 ± 0.1 mM).

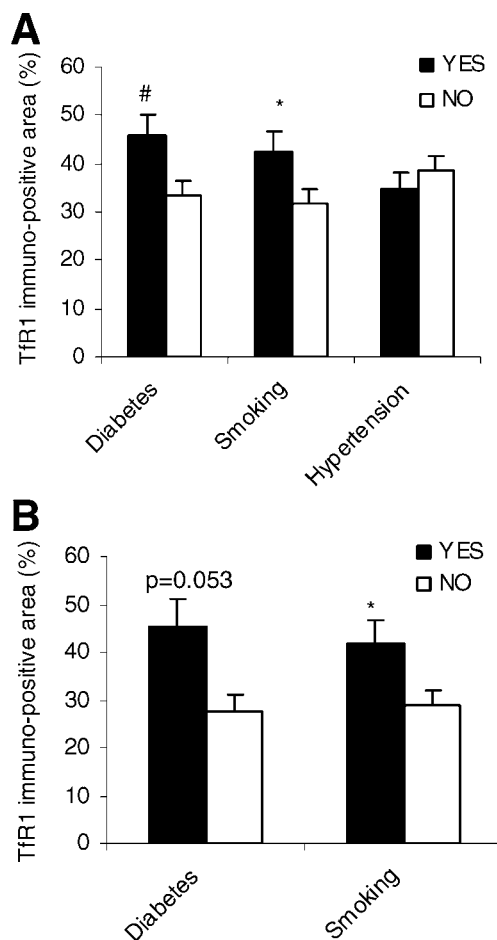


Figure 5. Smoking but not hypertension is significantly associated with higher levels of TfR1 expression in carotid atherosclerotic lesions. (A) Lesional TfR1 expression is significantly elevated in the patients being a smoker (* $P < 0.05$; yes = 19, no = 33). A trend for significant difference in TfR1 expression appeared in the patients with diabetes mellitus ($n = 9$) compared with the patients without diabetes ($n = 43$; # $P = 0.08$). No significant difference in TfR1 expression was seen between the patients with ($n = 39$) or without hypertension ($n = 13$). (B) Lesions from patients with diabetes but without smoking habit ($n = 6$) showed higher levels of TfR1 than ones from patients without diabetes and smoking habit ($n = 27$). Similarly, when lesions from patients with diabetes were excluded in both yes ($n = 16$) and no ($n = 27$) of smoking groups, a significant higher levels of TfR1 expression was seen in the lesions from patients with smoking habit.

Discussion

In this study, we reported that the TfR1 expression in atherosclerotic lesions is significantly associated with ferritin accumulation and macrophage infiltration, which contribute to development and rupture of human carotid atheroma. These findings are of relevance to the role of intralosomal iron metabolism in atherogenesis.

Under atherogenic pro-inflammatory conditions, TfR1 expression may be increased in atheroma cells, particularly in monocytes or macrophages due to the following possible reasons: Firstly, TfR1 expression may be a differentiation- or proliferation-related process. Transition of monocytes *in vitro* to macrophages causes TfR1 up-expression accom-

panied by high ferritin induction, suggesting that iron uptake and storage has a critical role in maturation of macrophages (17). Secondly, intraplaque hemorrhage, erythrophagocytosis, and apoptotic cell phagocytosis may result in accumulation of excessive cellular iron (5, 18) and influence TfR1 expression (19). Thirdly, pro-inflammatory cytokines including IL-1, IL-2, γ -IFN, and TNF- α modulate TfR1 regulation *via* different mechanisms (20–22). Fourthly, reactive oxygen species (ROS) may also affect TfR1 expression. ROS including H₂O₂ and O₂^{•-} which are both released by inflammatory cells modulate iron response protein 1 (IRP₁) in opposing directions. Extracellular H₂O₂ but not superoxide determines the compartment-specific activation of transferrin receptor by IRP₁ (23).

Increased expression of cathepsin L has been demonstrated in human atherosclerotic lesions (24), and cathepsin L participates in atherosclerosis by degrading elastin and collagen and regulating blood-borne leukocyte transmigration and lesion progression in mice (25). The correlations between expression of TfR1 and lysosomal cathepsin L seen in this study are intriguing and need further mechanistic explanations, although earlier studies have revealed that several subcellular events of macrophages in atherogenesis may require interactions of TfR1 and lysosomal cathepsin L. Firstly they may together participate in macrophage antigen processing. In murine macrophages, major histocompatibility complex II (MHC II)-peptide loading occurs in TfR-positive endosomes; MHC II-fluoresceinated complexes were only observed in TfR-positive organelles (26). Secondly, oxidized lipids under the influence of pro-inflammatory cytokines induce apoptosis or apoptosis-like cell death, in which the endo-lysosomal compartment and an iron-mediated pro-oxidant status may coordinately result in ectopic expression of both TfR1 and lysosomal cathepsins (6, 27). A nuclear localization of both cathepsins B and L has been demonstrated in human atheroma, which can be induced in 7-oxysterol-induced macrophage apoptosis (24, 27). Thirdly, overexpression of TfR1 in human carotid atheroma may reflect the dysfunction of foam cell lysosomes induced by oxidized lipids as we showed previously (24, 28, 29) and as further established by a recent study (13). It is generally accepted that under normal conditions the TfR complex is internalized in endosomes or lysosomes. As demonstrated in the present study, lesional cathepsin L predominately accumulates in the nuclear regions of atheroma cells. Ectopic expression of lysosomal cathepsin L may prevent internalization of TfR1 in the endosome-lysosome system, resulting in the overexpression of lesional TfR1 in human carotid atheroma.

Ferritin is considered an important intracellular anti-oxidant since it sequesters iron (mainly by the L-chain) and also has ferroxidase capacity (by the H-chain). However, endogenous ferritin, accumulated due to exposure to iron or lipids, is not sufficient to fully protect cells against oxidative stress (6). Under certain conditions ferritin can even release redox-active iron and thus may also function as a pro-

oxidant (12, 30, 31). Massive induction of ferritin in human atheroma lesions may be *via* the similar mechanisms as described above for TfR1 induction. However, haemin derived from hemoglobin degradation following erythrophagocytosis may be an important ferritin inducer in atherogenesis (32).

Because there is confirmed iron accumulation in human atherosclerotic lesions (33–35) in either redox-active form or low molecular weight form, we can further propose that parallel increase in ferritin and TfR1 expression in human atherosclerotic lesions is at least partly induced by increased cellular iron uptake, mainly in macrophages. This iron metabolism pathway may promote iron-catalyzed production of highly toxic hydroxyl radicals *via* the Fenton/Haber-Weiss reaction and LDL modification in atherosclerosis.

Expression of TfR1 and ferritin were increased in parallel with lesional macrophages and linked to vulnerable or ruptured plaques and severity of carotid atherosclerosis. TfRs have been explored as molecular targets for imaging and treatment of rapid growth tumors (36, 37). Our findings on TfR expression and abnormal iron metabolism in atherosclerotic plaque macrophages suggest potential targets for imaging the structure and severity of human atheroma at the cellular and molecular level.

Study Limitations. The study design is descriptive, and therefore the findings should be interpreted with caution with regard to a causal link between expression of iron-related proteins, lysosomal cathepsin L, and plaque appearance. Findings related to expression of iron-related proteins and plaque appearance, and iron-related proteins and clinical symptoms, may be in part attributable to the recognized association between plaque appearance and symptoms.

In conclusion, lesion-dependent TfR1 expression is significantly correlated with ferritin accumulation by macrophages in human carotid atheroma. These findings are consistent with the proposal (38) that pathologic macrophage iron metabolism may contribute to the development and rupture of human atheroma and their clinical symptoms.

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