Fn14 Is Upregulated in Cytokine-Stimulated Vascular Smooth Muscle Cells and Is Expressed in Human Carotid Atherosclerotic Plaques
Modulation by Atorvastatin

Begoña Muñoz-García, BSc; Jose Luis Martín-Ventura, PhD; Elena Martínez, MD; Santiago Sánchez, MD; Gonzalo Hernández, MD; Luis Ortega, MD; Alberto Ortiz, MD; Jesús Egido, MD; Luis Miguel Blanco-Colio, PhD

Background and Purpose—Interaction between different members of the tumor necrosis factor superfamily and their receptors elicits diverse biologic actions that are implicated in the pathogenesis of atherosclerosis. We have analyzed the expression of Fn14 and its ligand TWEAK in carotid atherosclerotic plaques and its potential modulation by atorvastatin in vivo. Furthermore, we have studied whether proinflammatory cytokines regulate Fn14 expression in human aortic smooth muscle cells (hASMCs) in culture as well as the potential regulation by atorvastatin treatment.

Methods—Fn14 and TWEAK expression was analyzed in human carotid atherosclerotic plaques. Furthermore, Fn14 expression was studied in hASMCs in culture.

Results—Fn14 and TWEAK are expressed in macrophages and smooth muscle cells in carotid atherosclerotic plaques. Proinflammatory cytokines (interleukin-1β and interferon-γ) upregulate Fn14 expression in hASMCs. This effect was prevented by atorvastatin treatment and reversed by mevalonate and geranylgeranyl pyrophosphate. Geranylgeranyl transferase inhibitor, toxin B (Rac and Rho inhibitor), C3 exoenzyme (Rho inhibitor), and Y-27632 (Rho kinase inhibitor) also decreased Fn14 expression, implicating the Rho/Rho kinase pathway in the regulation of Fn14 expression. Finally, atorvastatin treatment reduced Fn14 expression in vivo.

Conclusions—TWEAK and Fn14 are expressed in atherosclerotic plaques and could be novel mediators of atherosclerosis. Atorvastatin diminishes Fn14 expression in vitro and in vivo providing novel information of the beneficial properties of statins. (Stroke. 2006;37:2044-2053.)

Key Words: carotid arteries • HMG-CoA reductase inhibitors • inflammation • Rho GTP-binding proteins • smooth muscle cells

Atherosclerosis is currently described as an inflammatory disease, given that the main components of chronic inflammation are present in this process: cell recruitment, proliferation, neovascularization, and sclerosis. Vascular lesions are caused by inflammatory and fibroproliferative responses to injury of the endothelium and vascular smooth muscle cells (SMCs).

Interaction between members of the tumor necrosis factor (TNF) superfamily and their receptors elicits diverse biologic actions that participate in atherosclerosis development. These responses include the expression of adhesion molecules, proinflammatory cytokines, matrix metalloproteinases, and tissue factor, which are known to increase plaque instability. One of these members is the TNF-like weak inducer of apoptosis (TWEAK/TNFS12), which has different biologic functions, including induction of inflammation, activation of cell growth, and stimulation of apoptosis.2,3 The receptor of this protein is TWEAKR/Fn14, the smallest reported member of the TNF superfamily. TWEAK and Fn14 are expressed in different cell types, including endothelial and SMCs.4 In addition, TWEAK/Fn14 interaction induces the secretion of proinflammatory chemokines such as interleukin-8 (IL-8) and monocyte chemotactant protein-1 (MCP-1) in different cell types.5 Fn14 has been detected in the diseased vessel wall suggesting that TWEAK/Fn14 may be implicated in atherosclerotic plaque destabilization.6

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) have been shown to reduce cardiovascular events.7 Although the salutary effects of these agents may be explained by their beneficial actions on the
lipid profile, different lines of evidence suggest that they exhibit effects unrelated to lipid reduction.8

We have analyzed the expression of Fn14 and TWEAK in human carotid atherosclerotic plaques. Moreover, Fn14 expression and its regulation by atorvastatin in human SMCs vitro have been studied. Finally, we have examined the expression of Fn14 and its ligand TWEAK in carotid atherosclerotic plaques of patients treated with 80 mg atorvastatin per day in the context of a simple-blind, randomized trial.

Materials and Methods

In Vitro Studies: Human Aortic SMCs

Reagents
Ham’s F-12, penicillin, streptomycin, and trypsin-EDTA were obtained from BioWhittaker. Fetal bovine serum was from Gibco. Atorvastatin pure substance (calcium salt) was provided by Pfizer Inc. FTI-277, GGTI-286, toxin B, and C3 exoenzyme were from Calbiochem. Y-27632 was from Tocris. Recombinant human TWEAK was from Alexis. The remaining reagents were obtained from Sigma unless specified otherwise.

Cell Culture
Human aortic SMCs (hASMC) (ATCC; CRL-1999) were cultured in Ham’s F-12 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, ITS (2.5 µg/mL insulin from bovine pancreas, 2.5 µg/L human transferrin, 2.5 ng/mL sodium selenite), 30 µg/mL endothelial growth supplement, at 37°C in 5% CO2. Cells were used between passages 3 and 7.

Western Blot
Cells from different experimental conditions were collected and pelleted. Western blots were performed as previously described.9 The blots were incubated with mouse anti-human Fn14 antibody (ITEM-4; ebioscience) and rehierarchical with anti-α-tubulin monoclonal antibody (B-5 to 1–2; Sigma) to confirm equal loading and transfer of samples. Quantification was expressed as arbitrary densitometric units (ADU).

Immunoﬂuorescence Staining
Aortic SMCs were grown in four chambers of tissue cultured-treated glass slides (Falcon Labware). Cells were washed, fixed in methanol/acetone at −20°C, and incubated with 10% goat serum in phosphate-buffered saline for 1 hour. Mouse anti-human Fn14 (ITEM-4) was used as the primary antibody and a fluorescein isothiocyanate-labeled goat anti-mouse IgG was used as secondary antibody (Caltac Laboratories). Nonimmune serum or secondary antibody alone was used as the negative control. Cells were mounted in 70% glycerol. Analysis was performed with a Leica DM-IRB confocal microscope.

RNA Extraction and Real-Time Polymerase Chain Reaction
Total RNA was obtained by Trizol method (Life Technologies) and quantified by absorbance at 260 nm in duplicate. Real-time polymerase chain reaction (PCR) was performed on a TaqMan ABI 7700 Sequence Detection System using heat-activated TaqDNA polymerase (Amplitaq Gold). After an initial hold of 2 minutes at 50°C and 10 minutes at 95°C, the samples were cycled 40 times at 95°C for 15 seconds and 60°C for 60 seconds. GAPDH and 18S rRNA served as housekeeping genes and were amplified in parallel with the genes of interest. The expression of target gene was normalized to 18S rRNA. sMCP-1 expression was performed in duplicate in the context of a simple-blind, randomized trial.

Enzyme-Linked Immunosorbent Assay of MCP-1
Concentrations of sMCP-1 were determined in duplicate with commercially available enzyme-linked immunosorbent assay kits (R&D systems). Twenty microliters of supernatant sample was assayed in parallel to known standard concentrations. Each assay was calibrated using a sMCP-1 standard curve.

In Vivo Study: Human Carotid Atherosclerotic Plaques

Patients
Ten nonatherosclerotic mammary endarteries from patients undergoing cardiac surgery and 15 consecutive subjects undergoing carotid endarterectomy at our institution were included in the study. We studied atherosclerotic plaques in two different areas: shoulder and cap. The shoulder region was defined as the plaque area at both sides of the lipid core and the fibrous cap as the rim over the atheroma.10 Furthermore, 20 patients with carotid stenosis10 70%, who were prescribed by a vascular surgeon (Prof J. Serrano) to undergo carotid endarterectomy, were included in a simple-blind, uncenter, randomized trial to receive 80 mg atorvastatin per day (n=11) or usual care (n=9) during 4 to 6 weeks before surgery. Additional details on this trial have been previously described.11 Informed consent was obtained before enrollment in all cases. The study was approved by the Hospital’s Ethics Committee according to the institutional and the Good Clinical Practice guidelines.

Immunohistochemistry
Carotid atherosclerotic plaques were stored in parafomaldehyde for 24 hours and later in ethanol until paraffin-embedded. Immunohistochemistry was performed as previously described.10 Primary antibodies were mouse anti-human Fn14 (ITEM-1) antibody, rabbit anti-TWEAK antibody (sc-32254; SCB), anti-smooth muscle cell α-actin (1A4; Dako), or anti-human macrophages (HAM-56; Dako). For colocalization studies, immunofluorescence was carried out on slides after performing immunohistochemistry for macrophages, SMCs, or Fn14. Negative controls using the corresponding IgG were included to check for nonspecific staining.

Quantification
Computer-assisted morphometric analysis with the Olympus semi-automatic image analysis system Micro Image software (version 1.0 for Windows) was performed by a pathologist who was blinded to the patient’s group that the atherosclerotic plaques belong to, as previously described.10,11 Results are expressed as percentage of positive staining per millimeter squared.

Statistical Analysis
In vitro experiments were performed at least three times. Statistical analysis was performed with GraphPAD InStat Software. Results are expressed as mean±standard error of mean and analyzed by analysis of variance and Student t test. Differences were considered significant at P<0.05.

Results

Fn14 and TWEAK Expression in Human Carotid Atherosclerotic Plaque
TWEAK is expressed in nonatherosclerotic mammary arteries, whereas the expression of its receptor Fn14 is almost absent (Figure 1A). However, Fn14 and TWEAK are both present in human carotid atherosclerotic plaques, their expression being higher in the shoulder than in the cap region, although it did not reach statistical significance (Figure 1B). Furthermore, SMCs and macrophages express Fn14 (Figure 1C) and Fn14 and TWEAK are expressed by the same cells (Figure 1D).
Proinflammatory Cytokines Upregulate Fn14 Expression in hASMCs: Modulation by Atorvastatin

Because endothelial cells and macrophages constitutively express Fn14,3,6 the effect of proinflammatory cytokines on Fn14 expression in hASMCs was analyzed. IL-1β and interferon (INF)-γ increase Fn14 expression in a dose- (Figure 2A) and time-dependent manner (Figure 2B and 2C). The maximal effect was observed with 100 U/mL IL-1β plus 500 U/mL INF-γ at 24 hours. mRNA expression of Fn14 was incremented by IL-1β plus INF-γ in a time-dependent manner, peaking at 6 hours (Figure 2C). Moreover, atorvastatin treatment decreased Fn14 mRNA (6 hours) and protein expression (24 hours) induced by IL-1β-INF-γ in a dose-dependent manner (Figure 3A and 3B).

By confocal analysis, we have also observed that Fn14 expression was induced in hASMCs growing with IL-1β/INF-γ, which is inhibited by atorvastatin treatment (Figure 3C).

Mevalonate and GGPP Prevent the Effect of Atorvastatin on Fn14 Expression

Because mevalonate is the metabolite that is directly synthesized by the HMG-CoA reductase, we analyzed its potential effect on Fn14 expression in hASMCs. Cells were preincubated with 10 μmol/L atorvastatin and then incubated with IL-1β-INF-γ in the presence of 100 μmol/L mevalonate. Mevalonate reversed the inhibitory effect of atorvastatin on Fn14 mRNA (6 hours) and protein (24 hours) expression (Figures 3C and 4A).

Figure 1. Localization of Fn14 and TWEAK in human arteries. (A) Expression of TWEAK and Fn14 in healthy mammary arteries. (B) Expression of Fn14 and TWEAK in the shoulder and cap regions of carotid atherosclerotic plaques. (C) Colocalization of Fn14 (green) and macrophages or SMCs (brown). (D) Colocalization of Fn14 (brown) and TWEAK (green).
tionally, 5 μmol/L GGPP, but not 5 μmol/L FPP, reversed the inhibitory effect of atorvastatin on Fn14 expression (Figure 4A).

To further study the role of isoprenoids related to the mevalonate pathway in the regulation of Fn14 expression, two different inhibitors of isoprenoid transferases were used: farnesyl transferase inhibitor (FTI-277; 5 nmol/L) and geranylgeranyl transferase inhibitor (GGTI 286; 30 μmol/L). Only treatment with GGTI reduced the expression of Fn14 elicited by IL-1β/INF-γ (Figure 4B).

Rho as a Mediator of Fn14 Expression
GGPP is implicated in the transcriptional modification of small G proteins. For that reason, we have used two different inhibitors (toxin B or C3 exoenzyme), which inactivate proteins of the Rho family. Toxin B inhibits small G proteins such as Rho, Rac, and Cdc42 and C3 exoenzyme induces Rho inactivation. Treatment of hASMC with 1.25 ng/mL toxin B or 10 μg/mL C3 decreased Fn14 mRNA (6 hours) and protein (24 hours) expression elicited by IL-1β/INF-γ (Figure 4B).
These results suggest the involvement of Rho proteins in the regulation of Fn14. Furthermore, atorvastatin treatment diminished the presence of RhoA in the membrane with reciprocal increment in the cytosol (supplemental Figure, available online at http://stroke.ahajournals.org). Treatment with Y-27632, which induces a specific inhibition of the kinase activated by Rho (ROCK), reduced Fn14 mRNA (6 hours) and protein (24 hours) expression in a dose-dependent manner, indicating that the Rho-ROCK pathway regulates Fn14 expression (Figure 4C).
Atorvastatin Decreases MCP-1 Release Induced by TWEAK

Because the Fn14 ligand TWEAK can induce proinflammatory responses in different cell types, the potential effect of atorvastatin on TWEAK-induced MCP-1 release by hASMCs was explored. For this purpose, instead of culturing hASMCs with the proinflammatory cytokines used in the previous experiments (which saturates the MCP-1 levels), cells were cultured in the presence of different doses of fetal bovine serum (FBS).

In this context, FBS increased Fn14 expression in a dose-dependent manner (Figure 5A). Incubation of 0.1 µg/mL recombinant TWEAK in the presence of 2% FBS (minimal FBS dose that upregulates Fn14 expression; Figure 5B) increased MCP-1 secretion by hASMCs compared with 2% FBS alone (Figure 5C). Treatment with atorvastatin decreased MCP-1 release induced by TWEAK with FBS, which could suggest that atorvastatin can reduce the proinflammatory response induced by TWEAK/Fn14 interaction.

Figure 4. (A) Mevalonate (MVA) and GGPP reverse atorvastatin-mediated inhibition of Fn14 mRNA and protein expression. Real-time PCR and Western blot showing effect of atorvastatin in presence or absence of MVA, FPP, or GGPP. *P<0.05 versus control; †P<0.05 IL-1β/INF-γ; ‡P<0.05 versus atorvastatin. (B) GGTI, toxin B, and C3 decrease Fn14 mRNA and protein expression. Real-time PCR and Western blot showing effect of FTI, GGTI, toxin B, and C3 on Fn14 expression. *P<0.05 versus IL-1β/INF-γ. (C) Y-27632 decreases Fn14 mRNA and protein expression. Real-time PCR and Western blot showing effect of Y-27632 on Fn14 expression. *P<0.05 versus IL-1β/INF-γ. Results are expressed as mean±SEM of three independent experiments. (D) Schematic representation of the potential mechanism of action of atorvastatin on Fn14 expression.
Modulation of Fn14 and TWEAK in Human Carotid Atherosclerotic Plaques by Atorvastatin

Fn14 and TWEAK expression was analyzed in atherosclerotic plaques of 20 subjects who were randomized to receive 80 mg atorvastatin per day (n = 11) or usual care (n = 9) for 4 to 6 weeks before they underwent carotid endarterectomy (supplemental Table, available online at http://stroke.ahajournals.org). Plaques obtained from patients who received atorvastatin treatment showed decreased Fn14 expression relative to plaques from nontreated patients (Figure 6A). However, no changes were observed in TWEAK expression (Figure 6B). These results suggest that atorvastatin can regulate the expression of Fn14 in vivo.

Discussion

Different proteins of the TNF superfamily have been detected in human atherosclerotic plaques, including the novel member Fn14. We have observed that TWEAK is expressed in nonatherosclerotic arteries, whereas Fn14 expression is almost absent. Moreover, Fn14 and its ligand TWEAK are expressed in different areas of atherosclerotic plaques and these proteins colocalize with SMCs and macrophages. Moreover, Fn14 and TWEAK colocalize within the same cell, indicating that Fn14/TWEAK interaction could be present in diseased vessel wall, which may have adverse outcomes in atherosclerotic lesions. TWEAK/Fn14 interaction increases proliferation and migration and induces apoptosis in some inflammatory cells such as monocytes. However, when TWEAK was added in our in vitro experiments, no features of apoptosis in hASMCs were observed (not shown). TWEAK/Fn14 pathway can also upregulate the proinflammatory response observed in atherosclerotic plaques. Incubation of TWEAK with cells that participate in the development of atherosclerotic lesions induces the expression of chemoattractant proteins such as MCP-1 and IL-8. In addition, Fn14 induce matrix metalloproteinases-9 expression in macrophages, a metalloproteinase implicated in plaque instability.

We also have observed for the first time that a proinflammatory environment induced by cytokines upregulate Fn14 expression in hASMC. In this context, HMG-CoA reductase inhibitor atorvastatin treatment reduced Fn14 expression elicited by cytokines. Fn14 downregulation induced by atorvastatin was reversed by mevalonate, the metabolite that is directly synthesized by the HMG-CoA reductase, suggesting that metabolites from the cholesterol pathway downstream of mevalonate could be important for the regulation of Fn14. Some of the effects of statins are associated with the inhibition of isoprenoid synthesis. We tested the potential effect of GGPP and FPP on Fn14 expression, showing that only GGPP prevented the downregulation induced by atorvastatin. The reduction of Fn14 expression was also observed by adding GGTI to the culture, but not FTI, indicating the requirement of an intermediate product modified by geranylgeranylation through the geranylgeranyl transferase in Fn14 expression. GGPP is used...
for the posttranslational modification of important cell proteins, including small G proteins (Ras and Ras-like proteins: Rac, Rab, and Rho).\textsuperscript{18} Toxin B from \textit{Clostridium difficile}, which inhibits small GTPases (Rho, Rac, and Cdc42), reduced Fn14 expression elicited by proinflammatory cytokines, suggesting that small G proteins can regulate Fn14 expression. Moreover, \textit{Clostridium botulinum} C3 exoenzyme, which selectively inhibits the activation of Rho proteins, but not other small G proteins such as Cdc42 or Rac, also reduced Fn14 expression. In this regard, we have previously demonstrated that treatment with atorvastatin reduces the anchorage of Rho into the plasmatic membrane of rat SMCs,\textsuperscript{19} and now we verified that atorvastatin also diminished the presence of RhoA in membrane and increased its accumulation in the cytosolic fraction. These results indicate that the reduction of Fn14 elicited by atorvastatin is closely related to the inhibition of Rho. However, we cannot exclude the possibility that other GTP-binding proteins can also regulate Fn14 expression in hASMCs. The mechanisms by which Rho can regulate Fn14 expression remain undefined. Using a specific inhibitor of Rho kinase (Y-27632), Fn14 expression was also reduced, indicating that statins, through the Rho/ROCK pathway, may regulate Fn14 expression. In this respect, different effects related with HMG-CoA reductase inhibition have been associated with the Rho/ROCK signaling.\textsuperscript{20,21}

The physiological importance of Fn14 downregulation by atorvastatin could be the result of the fact that it also decreased the proinflammatory response induced by its ligand, TWEAK, in hASMCs. The presence of recombinant TWEAK in the culture media increased the release of MCP-1 to the supernatant effect prevented by atorvastatin. It has been reported that TWEAK binding to Fn14 activates the NF-κB signal transduction pathway,\textsuperscript{14,15,22} and this transcription factor is a key regulator of numerous inflammatory response genes, including MCP-1. Statins treatment also reduces NF-κB activation and MCP-1 expression in a rabbit model of atherosclerosis.\textsuperscript{23} In the same context, we have recently demonstrated that 80 mg atorvastatin per day reduces NF-κB activation and MCP-1 expression in human carotid atherosclerotic plaques.\textsuperscript{11} In these subjects, treatment with atorvastatin also reduces Fn14, but not TWEAK expression, in atherosclerotic plaques, suggesting that the reduction of MCP-1 could be associated with the inhibition of Fn14. The reduction of Fn14 in vivo by atorvastatin could be related to the inhibition of Rho observed in our vitro studies. However, atorvastatin reduced lipid concentrations in these subjects and
cells. Atorvastatin diminishes Fn14 expression in vitro and in vivo probably through the inhibition of Rho prenylation. Our results suggest that Fn14/TWEAK could be novel mediators of atherosclerosis. Furthermore, the regulation of Fn14 expression by atorvastatin provides additional information about the beneficial effects of the HMG-CoA reductase inhibitors.

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Disclosures
None.

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Figure 6. Atorvastatin decreases Fn14, but not TWEAK, expression in vivo. (A) Representative photomicrographs of carotid sections showing the localization of Fn14 in atherosclerotic plaques. (B) Representative photomicrographs of carotid sections showing the localization of TWEAK in atherosclerotic plaques.

Therefore we cannot exclude that the diminution of Fn14 expression could be the result of a low-density lipoprotein cholesterol-dependent effect of atorvastatin. Finally, the concentration of statins used in our in vitro experiments is in excess of the therapeutic concentrations ranges (0.5 to 5 μmol/L). However, treatment with 80 mg atorvastatin per day also reduces Fn14 expression in carotid atherosclerotic plaques, indicating that our in vitro results could be related to the in vivo situations. Nevertheless, our data must be interpreted cautiously given the small sample size and that it was not a placebo-controlled study. In addition, the precise role of Fn14 and its ligand in atherosclerosis need further investigation.

In conclusion, Fn14 and TWEAK are expressed in human carotid atherosclerotic plaques and proinflammatory cytokines induce Fn14 expression in human aortic smooth muscle


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